MONOGRAPHS ON THE PROGRESS OF RESEARCH IN HOLLAND DURING THE WAR

MONOGRAPHS ON THE PROGRESS OF RESEARCH IN HOLLAND DURING THE WAR

EDITORIAL BOARD

R. HOUWINK, DELFT; J. A. A. KETELAAR, AMSTERDAM

1. Achievements in the Field of Optics by A. BOUWERS.
2. Technological and Physical Investigations on Natural and Synthetic Rubbers by A. J. WILDSCHUT.
3. Contribution to the Physics of Cellulose Fibres by P. H. HERMANS.
4. Modern Development of Chemotherapy by H. VELDSTRA and E. HAVINGA; H. W. JULIUS and K. C. WINKLER.
5. Groundwater Investigations by W. F. J. M. KRUL and F. A. LIEFRINCK.
6. Nutrition Researches by B. C. P. JANSEN.
7. On the Effect of Gonadotropic and Sex Hormones on the Gonads of Rats by S. E. DE JONGH and J. H. GAARENSTROOM.

8—9. Investigations into Phytohormones and their Applications by V. J. KONINGSBERGER, H. A. VAN DER LEK, and H. VELDSTRA.

10. Experimental Embryology by M. W. WOERDEMAN and CHR. P. RAVEN.
11. Sex-Endocrinology of Non-Mammalian Vertebrates by J. J. DUYVENÉ DE WIT and L. H. BRETSCHNEIDER.
12. Chemical and Physical Investigations on Dairy Products by H. EILERS, R. N. J. SAAL, and M. VAN DER WAARDEN.

Continued on page 177

MODERN DEVELOPMENT OF CHEMOTHERAPY

BY

E. HAVINGA
H. W. JULIUS - H. VELDSTRA
K. C. WINKLER

ELSEVIER PUBLISHING COMPANY, INC.

NEW-YORK AMSTERDAM

1946

PRINTED IN THE NETHERLANDS BY
MEIJER'S BOEK- EN HANDELSDRUKKERIJ
WORMERVEER

FOREWORD BY THE EDITORS

The purpose of this Series on the Progress of Research in Holland during the war is to show the world that scientists in the Netherlands have remained active during the five years of German occupation. The publication of monographs by the most representative research workers was already planned in the first years of the war, as a token of the undaunted spirit of the Netherlands.

In spite of the ever-growing burden of oppression and starvation research was continued intensively in all directions. Most of the material now published in this series was, for obvious reasons, kept a secret during the war.

It being the Editors' aim to present these monographs as early as possible after the war, the majority of them were prepared whilst the war was still on. Authors were therefore expected to give mainly the results of their own investigations, without exhaustive reference to the Anglo-Saxon literature, which would not be available to them until after the war.

The hope is expressed that the publication of this series will further intensify the interest shown by the allied nations in the fate of the Netherlands, demonstrating, as it does, the whole-hearted preparedness of our nation to contribute to the progress of mankind.

The Editors wish to express their greatest admiration for the publishing house of Elsevier, which took very serious risk in preparing this series in wartime, when all activity on behalf of such international purposes was strictly forbidden.

The Editors,
Dr Ir R. Houwink
Dr J. A. A. Ketelaar

Amsterdam, on V.E.-day, 8th May 1945.

PREFACE

In the beginning of 1936, soon after the significance of the discovery of Prontosil was understood, the late Professor of Bacteriology in the University of Utrecht, L. K. WOLFF, was one of the first in Holland to become interested in the new drug and its possibilities. Soon other Dutch clinicians contributed their observations, which were followed by those of workers in University and Industrial laboratories.

With the outbreak of the war, and its consequent effect on Holland, research workers here had to depend on themselves and could not compare their findings with those of fellow workers abroad. Publication of results was confined to Dutch periodicals and reports of work abroad reached us only after long intervals. What work was done here and what our ideas were, on this most interesting field of modern chemotherapy, will be summarized in the following pages.

Foreign literature will be referred to only in so far as it has a direct bearing on Dutch research work.

In writing this review the task was divided as follows: In an introduction starting points and perspectives were outlined by JULIUS from the bacteriologists' point of view, supplemented with chemical data of HAVINGA and VELDSTRA, in such a way that all the authors can be considered to endorse this in general.

The chapter on the mechanism of the action of the sulphonamides and of p-aminobenzoic acid consists of two parts; whilst JULIUS and WINKLER start from the results of their mainly bacteriological investigations, HAVINGA and VELDSTRA are guided in the exploration of this field by physico-chemical methods.

Though it will become clear that both ways of argumentation lead to comparable views, it must be stated that this was not intentionally pursued. So points of difference will be observed, which will appear more clearly afterwards in the more detailed papers.

The chemical investigations were summarized by HAVINGA and VELDSTRA, those in the pharmacological-clinical field by JULIUS and WINKLER.

We are much indebted to Dr J. J. DUYVENÉ DE WIT (Research Laboratorium N.V. Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman en Pharmacia, Amsterdam-Meppel) for the editing of Chapter V on antibiotic substances.

Many thanks are also due to Prof. Dr H. J. BACKER (Groningen), Miss J. Josso and Dr. P. VAN DER MEER (Leiden), Dr H. VERHAGEN (Bussum) and Dr A. P. VAN DER WEY (Amsterdam), who, by personal communications or by providing for inspection manuscripts, ready for print, rendered it possible to include in our review the most recent data of their investigations.

May 1945, Amsterdam

Research Laboratorium
Combinatie N.V.' en Amsterdamsche,
Bandoengsche en Nederlandsche Kininefabriek

VELDSTRA, HAVINGA

Utrecht

Hygiënisch Laboratorium Rijksuniversiteit

JULIUS, WINKLER

CONTENTS

I INTRODUCTION 1

II ON THE MECHANISM OF THE ACTION OF THE SULPHONAMIDES AND OF P.AMINOBENZOIC ACID 10

§ 1 BACTERIOLOGICAL INVESTIGATIONS 10
- *a Historical development* 10
- *b Characteristic properties of sulphanilamide action (as compared with other antiseptics)* 11
- *c Action of sulphanilamide on bacterial metabolism.* . . 17
- *d Antagonists and activators* 22
- *e Discussion on the action of sulphanilamide* 35
- *f Discussion on the action of sulphanilamide-derivatives* . 43
- *g The problem of chemotherapeutic resistance* 55

Literature 61

§ 2 PHYSICO-CHEMICAL INVESTIGATIONS 65
- *a Electrometric titrations* 65
- *b Ultraviolet absorption-spectra* 70
- *c Surface-(interface-) activity. (polarographic investigations)* 74
- *d Experiments on monolayers* 85
- *e On the mechanism of the action of p.aminobenzoic acid and of the sulphonamides* 86

Literature 99

III CHEMICAL INVESTIGATIONS 104

§ 1 SYNTHESIS AND ACTIVITY OF SULPHANILAMIDE-DERIVATIVES AND OF RELATED COMPOUNDS 104

a Derivatives of guanidine 105
b Derivatives of salicylic acid 106
c Derivatives of pyridine 106
d Derivatives of pyrimidine 107
e Derivatives of thiazole 110
f Derivatives of pyrazole, selenazole, oxazole and isoxazole 110
g Derivatives of triazole and tetrazole 111
h Derivatives of naphthionamide 113
i Derivatives of 2-aminothiazole-5-sulphonamide . . . 114
j Sulphides 114
k Sulphones 114
l Derivatives of p.aminobenzoic acid 115
m Sulphonamide antagonists 115

§ 2 ACTIVITY OF "SPOT-TEST" REAGENTS 118

§ 3 TABULAR SURVEY OF THE INVESTIGATED COMPOUNDS 119

§ 4 BIOCHEMICAL INVESTIGATIONS 119
Action of sulphonamides on enzymes 119

§ 5 ANALYTICAL EXPERIMENTS 135
a Quantitative determination of sulphonamides 135
b Diazo-reactions 136
c Quantitative determination of p.aminobenzoic acid and of sulphonamides, separately in mixtures 137

Literature 138

IV PHARMACOLOGICAL, IMMUNOLOGICAL AND CLINICAL INVESTIGATIONS 143

§ 1 PHARMACOLOGICAL INVESTIGATIONS 143
a Distribution, excretion 143
b Cyanosis 144
c Antipyretic action 144
d Action on carbonic anhydrase 144
e Tissue metabolism 145

§ 2 IMMUNOLOGY 145

§ 3 CLINICO-THERAPEUTICAL RESULTS 145
a Streptococci 145
b Pneumococci 146
c Meningococci 146
d Gonococci 146
e Other micro-organisms 147
f Toxic reactions 148
g New compounds 149
h Local application of sulphonamides 150
i Comparative clinical investigation of a number of sulphonamides 151
Literature 152

V MYCOTHERAPY. INVESTIGATIONS ON ANTIBIOTIC SUBSTANCES 158

§ 1 INVESTIGATIONS ON EXPANSINE 158
Literature 174

I. INTRODUCTION

In February 1935 **Domagk** published in the "Deutsche Medizinische Wochenschrift", his now well known communication on the therapeutic results of "Prontosil" in the treatment of streptococcal infections. This substance, diaminobenzene-azobenzenesulphonamide, ushered in a new era in the history of therapeutics. Chemotherapy, almost forgotten since the discovery of atoxyl, then salvarsan, germanine, plasmoquine, atebrine, etc., was suddenly awakened from her apparent slumber. In no other field of scientific research have so many publications been issued as in modern chemotherapy. Clinical, biological, industrial, and even popular papers have published innumerable articles on this subject, and in the ten years that have elapsed a good deal of progress has been achieved.

To understand the state of affairs of to-day we must go back to the time when the first attempt to get an insight into the action of the new substance succeeded by the discovery of **Tréfouel** et al. (1935), who demonstrated that the simple substance p-aminobenzenesulphonamide, known since 1908 (**Gelmo**) and present in the molecule of Prontosil rubrum, showed the complete activity of the original but much more complicated substance. It was not the azo dye that acted, but it was contrary to all expectations the simple sulphanilic acid derivative.

But here again the first enigma of chemotherapy arose. The active principle was unlike any known antiseptic or germicide, since in vitro the substance seemed to be inactive, and so the mystery of its action on the organism present in the infected host presented itself.

The investigations of **Colebrook**, **Buttle** and **O'Meara** (1936) showed that on bacteria in vitro there did exist a direct action, only if small inocula were made on the in vitro media on which the drug was to be tested. This observation brings us at once to the very heart of the subject.

Bacteria are easier to study in vitro, than protozoa or spirochaetae, because of their more independent life, so here the possibility appeared of studying the direct action of a chemotherapeutic agent (though under special conditions) on the causative agent of disease.

As everywhere else the work in Holland on sulphanilamide and its derivatives was pursued along different paths.

Chemical and microbiological laboratories have contributed a good deal of original work. Chemists in University and Industrial laboratories synthesized an enormous variety of substances from which it could be expected or hoped that they would contribute to chemotherapeutical progress. As far as they came to our knowledge they are recorded (Chapter III), but no doubt our records are very incomplete. What has come to our notice must be a relatively small part of the work done. Probably the bulk of the work still remains in the archives of the laboratories. From what was published, we can conclude that the main object was the synthesis of heterocylic derivatives, analogous to sulphapyridine; it must be stated the results achieved were remarkable and showed a good deal of inventiveness. Some of the substances tested clinically have already brought unmistakable advantages in clinical use. They are better tolerated, are less inclined to give toxic reactions, and may produce an improvement in their therapeutic effect, chiefly by having a better therapeutical index.

Doubtless too, the chemical laboratories, aided by the biologist, have used a good deal of energy in trying to synthesize substances not directly related to the sulphanilic acid molecule. This means that there must have been an energetic search for new chemotherapeutic substances based on other principles. Work in this direction may lead at some time to new discoveries; from a scientific point of view it is to be regretted that, because of much reservedness about the results obtained, in our record no mention will be made of the position of affairs in this interesting field of research.

The biological laboratory has yielded its contribution to modern chemotherapy, and during almost the whole period of chemotherapeutic renaissance it has gone its own way. Some of the older series of experiments now seem to be overtaken, either by the researches of workers abroad or by our own experiences; they are nevertheless links in a logically composed chain, and we are for this reason justified in recording them. It also may be asked if WOODS and FILDES and also KUHN and his collaborators with their theories, have not said the last word on the subject.

In Chapter II it will be discussed that many of our results and conclusions can be of value apart from the p-aminobenzoic acid[1] hypothesis, may this be accepted or not. The bulk of our researches were done independently from the work that led to the p.a.b.a.-hypothesis. Taken together they will be seen to complete one another.

The physico-chemical side of the problem, rather undefinite in the beginning, presented itself in all its urgency from the moment that the extreme activity of p.a.b.a. as a sulphanilamide antagonist came to be noticed. It opened a most interesting outlook on the problem and was studied in two different laboratories. The results are recorded in Chapter II of this issue, but it must be emphasized that work at this moment is still in progress and far from complete.

Clinical work has been rich in experience (Chapter IV). The sulphonamides have been applied widely and, fortunately, with a good deal of critical sense. Indications and contra-indications have been noted, statistical data on successes and failures have been gathered, toxic reactions have been studied together with ways to avoid them or to keep them within bounds.

Some of the clinical investigations involved experiments on susceptibility, or, on the resistance of the special causative bacterial strains to the particular drug used and parallels were drawn with the clinical results. The problem of resistance either primary or acquired presented itself, and was studied with more or less satisfactory results.

Pharmacological and toxicological studies mainly have been interwoven in the clinical work. They bear upon distribution in the body and body fluids and upon excretion. Relatively little work has been done on animals in the experimental laboratories. The drugs all being relatively non-toxic allowed of direct observation in man. Experimental work on the influence of the substances on the hemopoetic system did not come to our knowledge; but some investigations were carried out on the hemochromogens.

---

[1] Further to be indicated as p.a.b.a.

To the most recent development of chemotherapy, i.e., the combating of pathogenic bacteria by means of antibiotic substances got from several micro-organisms, particularly fungi (mycotherapy), contributions have also been made by a number of investigators in the Netherlands.

It is interesting to note that the starting points of these researches are different from those of the English and American investigators, and originate from the observation of VAN LUIJK that micro-organisms from the soil are capable of producing substances, antagonistic to plant parasites. In Chapter V the results obtained up till now by one research group are reported.

The leading subject of our record of Dutch scientific work during the war on sulphanilamide and its derivatives was the question of their mode of action, in which chiefly sulphanilamide itself was studied. In this respect one may ask if it is justifiable to pay so much attention to sulphanilamide. Since the remarkable potencies of this substance were discovered, a great number of compounds has been synthesized. To a great extent, but not entirely, they have superseded the original drug, being successful in many cases where sulphanilamide failed. In our opinion all derivatives, and even the less narrowly related substances, such as diaminodiphenylsulphone, sulphinic acids, and the derivatives thereof, are in their action of essentially the same character as sulphanilamide. This holds true even for sulphanilic acid, so that perhaps we would be justified in considering this substance as the base of the new chemotherapy, were it not that sulphanilamide was first in the field before sulphanilic acid had attracted any attention.

It will be argued in Chapter II that the action of sulphanilamide on bacteria is characteristic, well defined, and fundamentally different from any disinfectant, antiseptic, germicide, or by what other name it may be called. All the sulphonamides synthesized afterwards show the same characteristics; they are, apart from undeniable advantages, elaborations of the same theme[1].

Thus, as to the problem of the mechanism of the action of sulphanilamide and its derivatives, sulphanilamide itself remains the central point of interest, the more so because its so very characteristic activity in vitro appears in concentrations that can be very well realized in vivo. And this again is a justification of the purely experimental and scientific studies on sulphanilamide action in vitro, sometimes looked upon by the clinician as a laboratory phantasy!

Apart from the therapeutical and especially the clinical improvement, the heterocyclic derivatives have also offered some purely scientific gain. Some of them, as a matter of fact, appeared finally to be totally devoid of antibacterial and therapeutical effect, in vitro as well as in vivo. This means not only that the addition of a heterocyclic nucleus failed to be an improvement, as was the case with sulphapyridine, but that some special substitution of the SO_2NH_2-grouping is able to annihilate the action of the sulphanilamide. This allows us to classify the derivatives into those showing an improved sulphanilamide action, those showing an unaltered and those with a diminished action. The scale is a gliding one. This remarkable feature will be discussed in both Chapter II and III of our review. It offered, rather unexpectedly, an insight into the mechanism of the action of the sulphonamides (adsorption and fixation on the bacteria), (compare page 45 and 81).

Sulphonamides then have some direct action on bacteria, but not on all of them. Their being effective or not, cannot be connected with any bacterial classification. In some more or less related organisms the one can be profoundly influenced (Bact. coli), while the other is highly refractory (Proteus). Some quite unrelated infectants like streptococci or gonococci on the one hand, and the virus of lymphogranuloma inguinale on the other hand are all influenced by the drugs.

---

[1] This does not hold true for Marfanil (p-aminomethyl-benzene-sulphonamide), which shows a different mode of action. Though chemically regarded as undoubtedly a "sulphonamide", it should not designated as such in the usual chemotherapeutical sense.

Some elementary bacterial insensitiveness to the sulphonamides does exist. Here the problem of specifity, at least specific affinity, of special derivatives of sulphanilamide, is touched on. Is it really true or only apparently so, that sulphathiazole has a reputation for combating staphylococcal infections, a power not attributed to other derivatives?

Further there can be an acquired resistance as is shown to be possible in streptococci, gonococci, pneumococci and others. In some kinds of bacteria e.g., *Bact. coli*, this resistance is temporary. In others, such as pneumococci, it seems, as far as can be judged from the limited period in which they could be studied as yet, to be permanent.

The question then arises if the elementary and the acquired form of resistance are of the same type and mechanism. And furthermore if the difference in the therapeutical effect of sulphanilamide on streptococcal and pneumococcal infections is merely of a quantitative character. Or, again, is the improvement that sulphapyridine has over sulphanilamide merely quantitative? As already mentioned, this must be answered partly in the affirmative. For some part, perhaps, there is a wider background in the introduction of the heterocyclic nucleus in the molecule (see Chapter II, page 53). This, too, must have bearing on biological differences between streptococci and pneumococci (Streptococcus viridans).

The acquired resistance has, more than probably at least, a direct relation to the sulphonamide action, for it is possible to evoke strains of pneumococci refractory to sulphapyridine by sulphanilamide, which do not differ from those made refractory by sulphapyridine itself.

A further important question is if the sulphonamides do kill the bacteria or if they are mere bacteriostatics. It is not yet clear how to answer this question. Under clear-cut experimental conditions bacteria are killed up to sterilization, even by concentrations that can be realized in vivo with not too great difficulty. But as a rule the "therapia sterilisans magna" in man is a pious hope. To qualify the action of sulphonamide as bacteriostatical is incomplete. On the other hand this qualification probably will be the best way to describe the situation in vivo. If the body, fighting against its invader, is left too quickly to its own forces, the effect of chemotherapeutic aid can be illusive. For a perfect result, integrity of the defensive powers of the body is of primary importance.

One of the main and essential properties of the sulphonamides, that sets them apart from all other antibacterials, is their relative inoffensiveness for the vital body mechanisms. This holds true even for the metazoal cells in vitro (tissue cultures). This points to the fact that the very point of attack of the substances is an essential link in bacterial (vegetable) metabolism, lacking, or at least unessential in animal metabolism. On this base the scope of the problem is at once essentially broadened.

Though the study of sulphanilamide action has — intentionally — been the central problem, some other questions in relation to the action of antibacterials have more or less incidentally crossed the path of our investigations. To give the sulphonamides their particular place, the general properties of germicides studied for comparison had to be examined by the special techniques used. It is not impossible that these researches will lead to a better understanding of the relationship between chemotherapeutics on the one side, and germicides, antiseptics, disinfectants etc, on the other.

From a chemical point of view the study of the sulphonamides and of p-aminobenzoic acid — the latter being an ergon of relatively simple structure and acting in very low concentrations — offered propitious perspectives for physico-chemical research. Just as with some other ergons we are puzzled as in this case, by the tendency of nature to make use of a simple and almost trivial chemical compound in the running of the wonderfully effective engines connected with life.

So that the organic chemist may obtain beneficial results for mankind by synthetic work in this domain, this furnishes moreover material that can be of great interest for the theorist.

Subtle variations in molecular structure, e.g., the displacement of a simple and "harmless" methyl-group, determine whether a compound be active or not, and here by comparison of a number of carefully chosen compounds it may be possible to discover the essentials hidden in these molecules.

It goes without saying that many theoretical speculations were made about the special nature of the properties of p.a.b.a. and the sulphonamides responsible for their physiological activity. Especially the newer conceptions about "resonance" between molecular structures and speculations about the influence of the ring-system on the basic and acid groupings in the molecules, play a dominant part and doubtless must be taken into consideration in future, when describing the rôle of this, and other, ergons in the living cell.

Nevertheless we can state that up till now the enigma of the mode of action of p.a.b.a. has offered serious resistance to all attempts to arrive at its final solution. On the other hand the interrelation p.a.b.a.-sulphonamides seems so promising a starting point, that our efforts must be continued with the greatest tenacity. Electrometrical titrations, measuring of absorption spectra and the studying of their behaviour in monolayers and at the mercury/solution boundary have offered useful information already (comp. p. 65 ff.); other physico-chemical methods will doubtless bring further data which will help to gain insight into this matter.

As one of the essential results we can put in the foreground that the surface-activity[1] of the sulphonamides is one of the determinant factors for the degree of their chemotherapeutical activity. This calls for our attention with respect to the displacement theory, where this adhesive power for a certain receptor, doubtless is an integral part of the speculations.

Another striking fact, noticeable in the mode of action of other ergons too, comes to the fore as soon as one realizes in how minimal a concentration the molecules of p.a.b.a. exert their influence. We are inclined to believe that the solution of the problem in this respect will be not far off as soon as the wonder of this effectiveness has been realized to its full extent (compare page 88 ff.).

---

[1] We should be inclined to use the term "adhesive power", were not this in use already for another, exactly defined, physico-chemical property.

There is (or has been?) some belief in a ratio in chemotherapy, rightly or wrongly based on the leading theories concerning the mechanism of the action of sulphonamides. But it is without real interest whether a theory has been right or not, if it has given rise to reflection, has stimulated research and perhaps has led to new possibilities. Then, despite its eventual defectiveness, it has justified its existence. For the time being, however, the answer to the question of rational chemotherapy is, that there is still much of a speculative element in the enormous variety of properties that endow substances with antibacterial potencies, not to mention the properties that would promote an antibacterial potency to a chemotherapeutic one. It will be necessary to stimulate the whole research on antibacterials in all its minor parts to arrive at a decision in the problem of chemotherapy. What EHRLICH gave us was a brilliant description of the problem, by which the pith of it could be understood. That is something quite different from resolving the problem.

It depends on personal appreciation if one considers the problem of modern chemotherapy can be solved by one theory — let us take it as proven — explaining one special feature, may it be ever so essential, or if one considers the problem of modern chemotherapy as one of large extension, bearing on many fields of bacterial, metaphytal and metazoal life, with outlooks on several practical and theoretical questions, in short as a problem of far-reaching possibilities, that will stimulate and fascinate for a long period.

II. ON THE MECHANISM OF THE ACTION OF THE SULPHONAMIDES AND OF P-AMINOBENZOIC ACID

§ I. BACTERIOLOGICAL INVESTIGATIONS

a. Historical development.

After the synthesis of prontosil rubrum by MIETSCH and KLARER (1935), the discovery of its chemotherapeutic activity in vivo by DOMAGK (1935),and the demonstration by TRÉFOUËL, TRÉFOUËL, NITTI and BOVET (1935, 1936) that p-aminobenzenesulphonamide was in fact the active substance, for some time — as in the early period of the anti-protozoal chemotherapeutics — the view was held that the drug was devoid of direct action on the parasites, as shown by its total inactivity as an antibacterial, when tested in the ordinary ways on germs in vitro.

This fact, combined with its exceptional chemotherapeutic activity in vivo gave rise to a number of theories, in which the mechanism of the action of these drugs was explained by an interference in the struggle between the host and the micro-organism, e.g., a stimulation of phagocytosis (FINKELSTEIN, 1938), an inactivation of bacterial toxins (LEVADITI and VAISMAN, 1935, 1938), or an inhibition of bacterial capsule-formation (LEVADITI and coll., 1935, 1937, 1938). Many of these conceptions, however, failed to be confirmed by most of the other investigators. Roughly speaking they disappeared (temporarily?) as the clue to the mechanism of sulphanilamide action.

COLEBROOK, BUTTLE and O'MEARA (1936) then demonstrated the bactericidal action of sulphanilamide in vitro, only however, when the number of bacteria inoculated into the medium containing sulphanilamide was small. The conception of sulphanilamide as a "weak" antiseptic, which only "sterilized small numbers of bacteria" was based on these observations, but quite erroneously, as afterwards the described effect appeared within a wide range to be independent of the sulphanilamide concentration.

b. Characteristic properties of sulphanilamide action (as compared with other antiseptics).

Wolff and Julius (1939), analyzing the action of sulphanilamide in vitro by hourly determinations of the number of viable bacteria (streptococci), met with some very characteristic phenomena and insisted on the fact, that these phenomena are essential properties of the drug's action.

1. With sulphanilamide, sterile cultures are obtained only — when the inocula are small — in media which permit bacterial growth.

2. For streptococci an action on the bacteria is obtained only in the presence of (horse)blood, (an enhancing action of blood was indeed demonstrated, see below) which is the more striking, considering the deleterious effect of blood (and protein-containing fluids in general) on the action of other germicides.

3. Cultures with sulphanilamide show a peculiar growth curve characterized by three features (Fig. 1);

α. The action of sulphanilamide — even with maximal concentrations — becomes manifest only after some hours (the so-called lag-time of action!). During that time (first phase of the curve), the cocci, seemingly unaware of the presence of sulphanilamide, multiply just as rapidly as in control-cultures.

β. In the second phase, multiplication slows down, and, dependent on the concentration of sulphanilamide a partial or total inhibition of growth (bacteriostasis) is obtained (as is the case with a definite, mostly rather low concentration of many a germicide).

γ. With slightly higher concentrations of sulphanilamide (about 0.01 % for streptococci, 0.1 % for *Bact. coli* in a synthetic medium, see Fig. 14, p. 45) the curve shows a third phase, i.e., the number of viable bacteria decreases gradually *until sterilization* is obtained. Though incidentally a delayed growth (of sulphanilamide-resistant forms) can be demonstrated after some days, a sterilization of the culture is the rule. We want to stress this point here, as even in recent literature (e.g., HIRSCH, 1942, 1943) the sterilizing action of the drug is doubted, mostly by authors using large inocula and taking turbidity or respiration as a measure of bacterial viability.

This peculiar form of curve will be found with an adequate technique only: i.e., estimating the number of viable bacteria by plating or some other improved method (JULIUS, 1938). In many studies on this subject the turbidity of the culture, estimated with nephelometer or extinctiometer, is taken as a measure of bacterial growth. Apart from the fact that living and dead bacteria add equally to the turbidity, it is evident that the peculiar "maximum" in the curve will be missed when turbidity is taken for growth, as turbidity only begins to be perceptible if about 10^6 bacteria per ml are present, whereas the cultures containing sulphanilamide, even at the "maximum", seldom do contain so many bacteria.

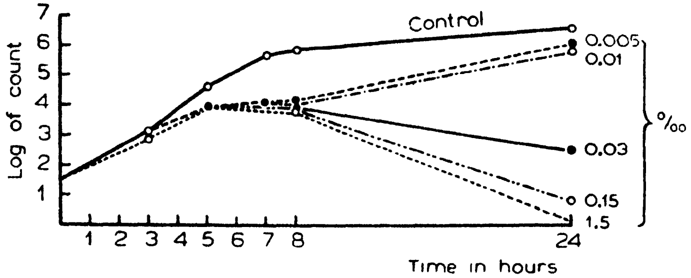


Fig. 1. Influence of various concentrations of sulphanilamide on streptococci in 50 proc. horse-blood broth.

Turbidity accounts only for what is in fact the end of the growth process (a, in Fig. 2).

With nephelometric technique no differentiation is possible between p, q, and r. The belief that cultures with sulphanilamide do not become sterile is largely due to considering such concentrations as being active, in which the medium keeps clear after 24 or 48 hours, overlooking that "clear" media may contain between nought and a million viable bacteria per ml.

Furthermore growth-retarding effects which are small in our view, as e.g., Curve 5 or 6 in Fig. 3 A, will be unduly magnified with a nephelometric technique (Fig. 3 B).

As with bacteria, which are rather resistant towards sulphanilamide, still a rather high activity (though not sterilization) seems to be observed using nephelometric technique. Experiments of this kind laid further stress on the view that sulphanilamide acts bacteriostatically and not bactericidally.

With regard to chemotherapeutical activity in vivo, it seems more important to consider small numbers of bacteria per unit of volume, and the first stages of their growth, than to study the millions per ml, which are estimated in turbidity experiments.

In considering the literature on this subject it has to be taken into account that in consequence of the facts mentioned above, there are essential differences in what is called "sulphanilamide action". We wish to define here as partial action: when growth is retarded (often after a lag-time of action), and as complete action: when this is followed by a dying off of the bacteria.

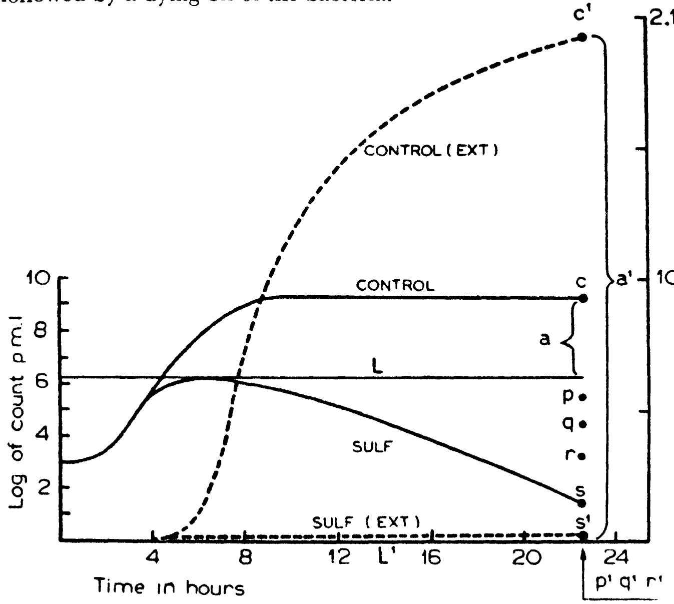


Fig. 2. Influence of sulphanilamide on bacterial growth: results of nephelometric estimations of turbidity, as compared with colony counts (half-schematically).

L = level at log of count about 6, below which the medium remains clear.

a = range (log of count-ordinate) in which turbidity can be measured. This range is magnified in the nephelometer scale from a to a′.

L, a, s, p, q, r refer to the log of count-ordinate,

L′, a′ etc. to the nephelometer.

The peculiar form of the "growth" curve (or rather "death" curve) was demonstrated by us to be typical for various bacteria, in various media, for all sulphanilamide derivatives studied. This form of curve was never obtained with any other germicide (barring atoxyl, which seems to be related to the sulphonamides in this respect). In Fig. 4 e.g., the action of 2 % phenol is compared with that of 0.15 % sulphanilamide.

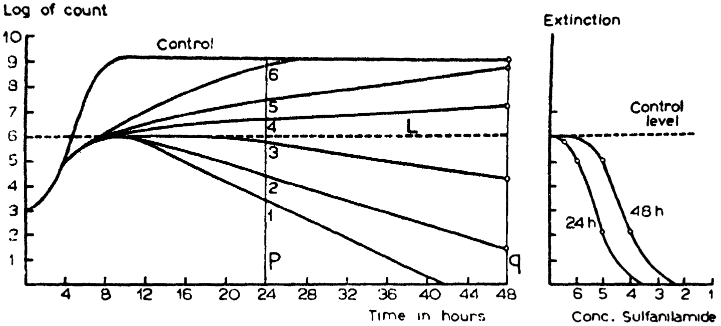


Fig. 3. Influence of various concentrations of sulphanilamide on bacterial growth, measured:
A with our improved plating method } schematically.
B with the nephelometer }
The estimations with the nephelometer are taken at 24 and 48 hours (vertical lines p and q).
L = level below which the media remain clear.

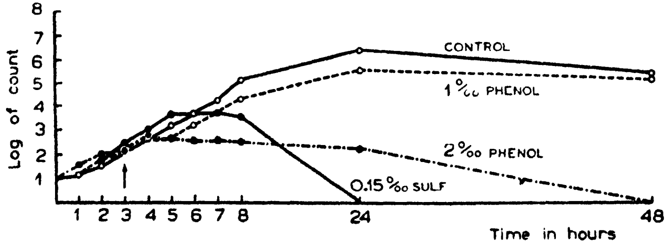


Fig. 4. Comparison of the influence of sulphanilamide and phenol on bacterial growth.

Though after six to ten hours phenol appears to be more active than sulphanilamide, the culture with sulphanilamide is sterilized in 24 hours, whereas with phenol this effect is obtained only after 48 hours.

Any theory of sulphanilamide-action will have to take account of this peculiar shape of the growth curve.

4. The lag-time of sulphanilamide action, i.e., the time during which the bacteria in the culture with sulphanilamide multiply just as rapidly as in the control cultures, was explained at an early date by Finklestone-Sayliss, Paine and Patrick (1937) by a specific action on young bacteria. Wolff and Julius (1939) invalidated this view by adding sulphanilamide to the growing cultures at different times after inoculation (Fig. 5), thus showing that the lag-period of action and the maximum in the curve of cultures with sulphanilamide, occurred in every case and independent of the age of the cocci.

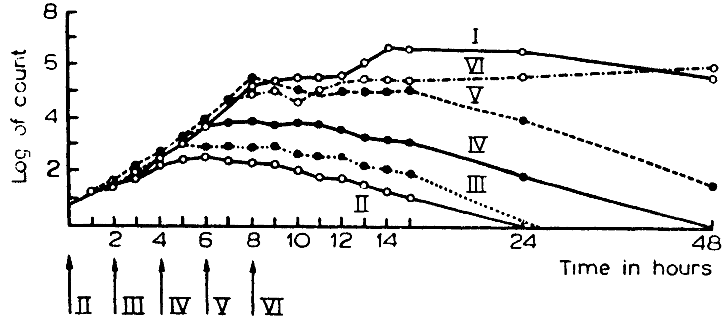


Fig. 5. Addition of sulphanilamide after different periods of growth. Streptococci; horse-blood broth; 0.015 % sulphanilamide added after 0, 2, 4, 6 and 8 hours.

Furthermore the lag-period and the height of the maximum (taking the moment at which sulphanilamide was added as zero-level) were the same in every case, indicating thereby that a constant number of cell divisions (about 8 in the case of Fig. 5) had to take place before growth inhibition by sulphanilamide set in.

Wolff and Julius (1939) concluded that the mechanism of sulphanilamide action somehow affects cell division or growth.

If the number of bacteria in the culture is less than 8 cell divisions from its saturation at the moment when sulphanilamide is added (Curve VI, Fig. 5), sulphanilamide action cannot be detected, which seems to strengthen the foregoing *conclusion and eventually explains why sulphanilamide is inactive with larger inocula. This early view still holds true, but being a special sample of a more general phenomenon, it had to be extended. The details will be discussed afterwards.*

Extending the work of WOLFF and JULIUS we devised the following experiment:

A small number of Bact. coli was inoculated

a. in a synthetic medium (K_2HPO_4, glucose and ammonium sulphate in tap water) with or without sulphanilamide, and

b. in the same medium, devoid of nitrogen by omitting the ammonium sulphate.

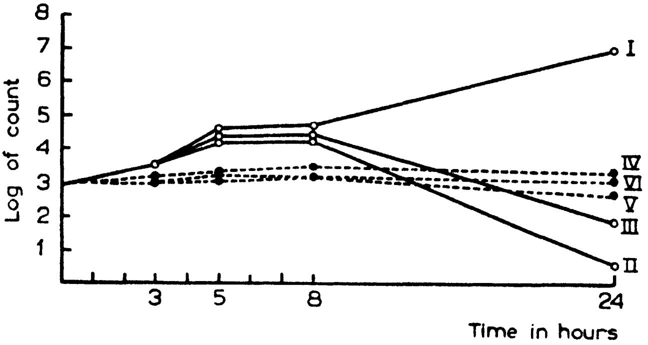


Fig. 6. Action of sulphanilamide on Bact. coli in a synthetic medium with and without a source of nitrogen (ammonium sulphate).
I, II, III with ammonium sulphate
IV, V, VI without ammonium sulphate
I and IV, control
II and V, with 0.1 % sulphanilamide
III and VI, with 0.02 % sulphanilamide.

The experiment in Fig. 6 showed that sulphanilamide does not harm the bacteria in the N-free medium in any way. The addition of sulphate of ammonia, however, promptly gave rise to a sterilization of the culture in about 24 hours. In this case sulphate of ammonia seems necessary for the action of sulphanilamide to become manifest. Evidently only when multiplication (or growth) has occurred, sulphanilamide is "active".

Here, as will be discussed more amply afterwards, an interference of sulphanilamide with growth (N-anabolism) seems the only way to explain the facts observed.

5. Julius and Winkler (1941 a), considering the many theories about an alteration in the sulphanilamide molecule before the action (another possible explanation of the lag-period of action), published some experiments, which showed beyond doubt that the sulphanilamide molecule is not altered before action, but acts as such. (For details see the original).

Summing up, the characteristic features of sulphanilamide action in vitro — at the same time a basis for further research — seem to be:

1. Sulphanilamide is active in vitro.
2. The drug acts directly on the bacteria.
3. No alteration of the drug occurs before action.
4. Addition of blood (see page 34) does not decrease sulphanilamide action but seems even to enhance its effect.
5. The activity in vitro is obtained with small inocula and actively multiplying bacteria only (see however page 38)
6. A typical form of "growth" curve appears.

4, 5 and 6, combined with the experiments under **4** (page 15) seem to indicate that cell division or growth is involved in the mechanism of sulphanilamide-action. In any case those three features put the sulphonamides quite apart from all germicides previously known.

c. The action of sulphanilamide on bacterial metabolism.

The characteristic features of the action of sulphanilamide, which is not directly bactericidal and different from any known antiseptic, has led to the general supposition of an interference of sulphanilamide in some part of bacterial metabolism. From the beginning we tried, by a systematic study of the influence of sulphanilamide on various parts of bacterial metabolism, to approach the solution of the problem of the mechanism of the action.

a. Sulphanilamide and catalase.

Locke, Main and Mellon (1939) supposed an inactivation of bacterial catalase by sulphonamides. The growth inhibition then would be due to the resulting accumulation of H_2O_2. By studying catalase activity with Photobacterium Fisheri as an oxygen indicator, we showed (Julius and Winkler, 1941) that sulphanilamide does not inactivate the catalase from blood or the bacterial catalase from Bact. coli and Streptococcus haemolyticus, nor does it affect the production of catalase in the growing cultures (first phase!).

Furthermore the characteristic action of sulphanilamide on Bact. coli and Streptococcus haemólyticus is maintained under anaerobic conditions (Winkler and Julius, 1941). This is incompatible with the supposition of Mellon and collaborators, as in anaerobic cultures no peroxide can be formed. The theory of catalase inactivation could thus be definitely dropped.

β. Sulphanilamide and bacterial respiration.

Winkler (1942) did not find that sulphanilamide had any influence on the oxygen consumption of resting Bact. coli and Str. haemolyticus, even when both organisms were grown in media containing sulphanilamide, in concentrations, which were actively bacteriostatic. (To obtain sufficient material, in the latter case a very large volume of medium had to be used, centrifuging at the moment when multiplication had stopped, but before the bacteria died off). The dehydrogenation velocity for various hydrogen donators was equally unaffected by sulphanilamide. Our results appeared to be confirmed recently by several authors (Hirsch, 1942; Söhring, 1942; Frei, 1942; Kohn and Harris, 1941). Punt (1943) also did not observe any influence of sulphanilamide in oxidations in vitro of brain and liver suspensions. With regard to a number of papers on this subject, which do not always lead to the same conclusions as did our findings, we like to put forward the following considerations:

a. As sulphanilamide acts on fast growing cultures only, whilst the action is delayed for some hours, experiments with resting bacteria, covering some hours only, can hardly be considered as conclusive.

With growing cultures, only experiments in which the O_2-consumption is calculated per bacterium can be of value.

b. For some sulphonamide derivatives a reduction of O_2-consumption was established beyond doubt. It is quite possible that here we deal with another action of the molecule.

c. The lack of influence on O_2-consumption in cases where sulphanilamide was actively inhibiting growth, as in our experiments, indicates that reduction of O_2-consumption, as was found in other cases, cannot be the actual cause of the growth inhibition.

The lack of influence of sulphanilamide on bacterial respiration is in accordance with the absence of any action on resting bacteria, and on cultures with large inocula which, as a matter of course, consume oxygen.

Taking into account the work of BARKER (1936), GIESBERGER (1936) and CLIFTON (1937), the absence of any action on bacterial respiration (carbohydrate-catabolism) might also exclude an influence on bacterial carbohydrate-anabolism. Thus an influence on carbohydrate metabolism in general seems improbable.

γ. *Sulphanilamide and bacterial nitrogen metabolism* (WINKLER, 1943).

a. Catabolism; hydrolytic enzymes.

No influence of sulphanilamide on gelatine hydrolysis by papaine or filtrates of Chr. prodigiosum or Ps. pyocyaneus could be detected, though sulphanilamide was known to be actively checking growth in both strains.

b. Nitrogen anabolism.

The preferential action of sulphanilamide on proliferating bacteria leads to the assumption that the action of sulphanilamide is closely related to the most essential part of growth and/or of cell division, i.e., the anabolism of bacterial protein, thus, with protein synthesis.

For the sake of convenience we have divided this process in three stages:

1°. synthesis of simple amino acids from inorganic N compounds:

$$R\cdot CO\cdot COOH + NH_3 + 2H \rightarrow R\cdot CH(NH_2)\cdot COOH + H_2O.$$

2°. synthesis of all kinds of other amino acids by transamination.

3°. synthesis of polypeptides and finally of proteins.

For some preliminary insight into the first two stages we were able to show (WINKLER, 1943):

1. That sulphanilamide does not interfere with the synthesis of aspartic acid from fumaric acid and ammonium chloride as described by QUASTEL and WOOLF (1926).

2. That sulphanilamide does not interfere with the synthesis of amino acids from inorganic nitrogen. We arrived at this conclusion by the following experimental results.

The addition of single amino acids to a culture of Bact. coli in a synthetic medium does not counteract sulphanilamide, which would be the case if the essential action of sulphanilamide was to interfere with the synthesis of that particular amino acid.

The addition of single amino acids only slightly accelerates growth, a marked acceleration being obtained only by adding various amino acids simultaneously. But even the addition of such an optimal mixture of amino acids did not exert any influence on the characteristic action of sulphanilamide. As the supply of a number of ready amino acids appears to save an important output of energy on the part of the bacteria (as can be read from the growth acceleration), from the undisturbed sulphanilamide action again we must conclude, that this action cannot be due to interference with the normal synthesis of amino acids from inorganic nitrogen. This does not mean that it could not interfere with the synthesis of some special amino acid, which becomes clear if one realizes, what would have happened, if p.a.b.a. had been included in the mixture!

3. That sulphanilamide does not interfere with the transamination: pyruvic acid + glutamic acid → alanine + ketoglutaric acid, by guinea pig muscle preparations.

4. That sulphanilamide does not interfere with the synthesis of hippuric acid anilide (BERGMANN) by papaine. (However, even a great number of experiments of this kind, with the same negative result, would not show that sulphanilamide does not interfere with some dipeptide synthesis by bacteria).

From the experiments mentioned in this section we will, until we get further evidence, derive the conclusion that sulphanilamide does not interfere with the synthesis of amino acids nor with transamination.

δ. *Discussion.*

Considering the experiments in the previous sections, the action of sulphanilamide cannot be explained by an interference with bacterial respiration, nor with the synthesis of amino acids, or with transamination. Per exclusionem we thus are inclined to suppose that sulphanilamide interferes with protein anabolism somewhere in the synthesis of proteins from their radicals (the amino acids?).

The analysis of the "growth curves" (see page 16), which led us to suppose an interference of sulphanilamide with growth, or better multiplication, seems in the first instance to substantiate this view.

An interference with a single step in the unknown series of reactions of protein synthesis seems not to be able to explain the action of sulphanilamide to its full extent. The inactivation by sulphanilamide of some bacterial enzyme-system, necessary for growth (anabolism) only, might explain the growth inhibition but never the "dying off" of the bacteria, indicated by the third phase of our "growth curve".

On the other hand, the inactivation of some bacterial enzyme system, related to bacterial maintenance (catabolism), might only explain the extermination of the bacteria, but would necessarily imply an action on large inocula or on resting bacteria as in the case of ordinary germicides.

To explain this incompatibility one might suppose the mechanism of sulphanilamide action to be complex, e.g., by interfering with several bacterial functions simultaneously. Still, considering the narrow interrelation of anabolism and catabolism (the terms anabolism and catabolism are used for convenience only, we are fully aware that interdistinction finally is not real) it would be sufficient to suppose an influence on the anabolism, which is then necessarily followed by a change in catabolic processes. Sulphanilamide might e.g., interfere with the production of a substance (enzyme or catalyst) which is necessary for bacterial maintenance. In that case growth will be inhibited because the substance is not formed, and the bacteria die off in the third phase because the supply of this substance runs out (see also page 39).

In the theory of L. W. JANSEN (1939), which supposes cell proteins to be formed on nucleoproteid-matrices, this might mean an interference with the formation of bacterial nucleoproteins. Further experimental work is this direction seems important.

Summary.

1. Sulphanilamide does not inactivate bacterial catalase.

2. Bacterial respiration, measured by O_2-consumption and dehydrogenation, is not influenced by sulphanilamide action. In any case it cannot be the explanation of sulphonamide action.

3. The synthesis of amino acids by bacteria, and transamination proceed normally in the presence of sulphanilamide. The possibility that sulphanilamide interferes with bacterial protein anabolism is discussed.

d. Antagonists and activators.

a. Antagonists.

p-Aminobenzoic acid.

In 1940 WOODS revealed the important fact, that a substance present in yeast extract was able to counteract sulphanilamide and to restore bacterial growth. The substance was supposed, and afterwards proven to be p-aminobenzoic acid. Minute quantities of p.a.b.a. inhibit the action of sulphanilamide, one molecule of p.a.b.a. antagonizing 10.000 molecules of sulphanilamide in a given case.

MÖLLER and SCHWARZ (1941) produced evidence that the ratio between the concentration of sulphanilamide and the just neutralizing concentration of the antagonist kept constant for various sulphanilamide concentrations.

Displacement of sulphanilamide by adsorption (or some other kind of bond) of p.a.b.a., seems the only probable explanation of the antagonism. Indeed p.a.b.a. is strongly absorbed by living bacteria, whereas sulphanilamide (within the experimental error) is not (JULIUS and WINKLER, 1942). As p.a.b.a. is not adsorbed by dead bacteria or by red blood cells or liver cells, a specific property of living bacteria seems to be involved here.

Shortly after the discovery of the antagonism between sulphanilamide and p.a.b.a. RUBBO and GILLESPIE (1940) showed that p.a.b.a. was a growth factor for Clostridium acetobutylicum, whilst KUHN and SCHWARZ (1941) showed the same for Streptobacterium plantarum. The growth promoting property of p.a.b.a. was very soon connected with the antagonizing action, and the fact that p.a.b.a. displaces sulphanilamide gave rise to the supposition that sulphanilamide might act by displacing p.a.b.a. (WOODS, 1940). FILDES as early as 1940 suggested, within the scope of a more general hypothesis on germicide action (Hg – SH-systems, etc.), that sulphanilamide inactivates an essential co-enzymic grouping of the susceptible organism, and that this grouping is most probably p.a.b.a. At the moment the inference appears to be that the growth factor p.a.b.a. is the prosthetic grouping of an essential bacterial ferment. The equilibrium: apoferment + p.a.b.a. ⇄ holoferment, is disturbed by sulphanilamide which, in virtue of its structural analogy, is adsorbed or bound to the apoferment in the place of p.a.b.a., rendering it inactive. In other words it is supposed that sulphanilamide activity is due to a displacement of p.a.b.a.

This very interesting theory was reinforced by a great number of important observations, e.g.:

1. The finding of similar antagonisms (SNELL, 1941; KUHN, WIELAND and MÖLLER, 1941): pantothenic acid/sulphopantothenic acid.

2. The discovery of other molecules with an analogous structure, showing sulphanilamide action, e.g., 4,4′-diaminobenzophenone. (KUHN, MÖLLER, WENDT and BEINERT, 1942).

3. The fact that sulphanilamide-resistant strains of bacteria (e.g., staphylococci) produced more p.a.b.a. than the original susceptible strains did.

The theory soon attracted attention in every field of biological chemistry and quite a number of antagonisms were rightly or wrongly explained in the same way.

The direct argument, however, that sulphanilamide acts by displacing p.a.b.a. is still lacking. Any antiseptic will annihilate the growth-promoting properties of p.a.b.a., i.e., check growth. That in the case of sulphanilamide growth can be re-established by the addition of more p.a.b.a. only shows again that p.a.b.a. antagonizes sulphanilamide. It cannot be seriously denied that the conception that sulphanilamide acts by the displacement of p.a.b.a., i.e., the opposite of the fact observed, in reality is nothing but a working hypothesis. We must ask the question if identifying the growth promoting and the antagonistic properties of p.a.b.a. is not the same as confusing them.

Indeed the quantitative relationship $C_{\text{sulphonamide}}: C_{\text{p.a.b.a.}}$ found by KUHN does not hold for the lowest sulphanilamide concentrations; there is a gap between growth promoting and antagonistic p.a.b.a. concentrations.

In a previous paper (JULIUS and WINKLER, 1942) we referred to some minor observations, which by the above theory could be explained with difficulty only.

1. The action of sulphanilamide on spirochaeta recurrens was said not to be neutralized by p.a.b.a. (FELDT, 1941). As in this case the action of sulphanilamide seemed not to be due to an antagonism towards p.a.b.a., at that time, we argued that this antagonism could not be the essential mechanism in other cases either. MUDROW and BOCK (1943), however, showed with FELDT's strain of spirochaeta recurrens, that p.a.b.a. did antagonize sulphanilamide. So the action on spirochaeta recurrens ceased to be an exception and an argument can no longer be drawn from it.

2. MANN and KEILIN (1940) observed, that sulphanilamide specifically inhibits carbonic anhydrase. The SO_2NH_2 group is the active grouping in this respect, benzenesulphonamide and $NH_2SO_2NH_2$ being active whilst sulphapyridine and other growth inhibitors with substituted SO_2NH_2 grouping are not. The activity of sulphanilamide as a growth inhibitor on the other hand depends on the amino group, essential substitutions here result in a loss of activity; $NH_2SO_2NH_2$ does not inhibit bacterial growth. Thus the bacteriostatic action of sulphanilamide and its inhibition of the carbonic anhydrase seem to be two different but specific properties of the same molecule. According to VAN GOOR (1942), p.a.b.a. at least counteracts partially the inhibition by sulphanilamide of the carbonic anhydrase of blood in vitro. It is

somewhat improbable that p.a.b.a. might be a prosthetic grouping for this enzyme as well as for the enzyme involved in bacterial growth. If it were, it still seems difficult to understand why sulphapyridine etc., would not inhibit the carbonic anhydrase, nor why $NH_2SO_2NH_2$ would not inhibit bacterial growth. At the time we argued that p.a.b.a. will compete with sulphanilamide on two different substrates, while acting through two different ways. It might, without being a prosthetic grouping, by means of its closely related structure displace the sulphanilamide in both cases and, missing the strongly polar SO_2NHR grouping, miss the disturbing properties of sulphanilamide and $NH_2SO_2NH_2$ in the one case and of sulphanilamide, sulphapyridine etc., in the other. Since the results of VAN GOOR's experiments were published in detail (1942) it became evident that the ratio p.a.b.a./sulphanilamide is of another order of magnitude in the case of the carbonic anhydrase. This fact suggests other explanations for some of the discrepancies mentioned and our former argument loses something of its strength.

It seems a rather thankless task to hold that the final proof of the above theory of sulphanilamide action has not yet been given. In spite, however, of the general approval this attractive hypothesis has met with, we want to point out the danger of using this hypothesis as a passe-partout, as has happened in other cases of antagonism where investigations have not gone any further. So it seems not superfluous to say that, even when accepting the theory, the function of p.a.b.a. in bacterial metabolism still will have to be elucidated before the problem of sulphanilamide action and its characteristics can be understood.

Peptone.

Like any chemical reaction, the process in bacterial metabolism, disturbed by sulphanilamide action, will be susceptible to any change of medium. Various inhibitors and activators thus may be expected. It is evident that p.a.b.a., active in so minute quantities, takes up a position of its own; still the other inhibiting or activating factors might give a clue to the kind of reaction involved. They are to be considered in that light even when at the moment we do not understand their meaning.

Apart from p.a.b.a. there is another substance from which it is known, that it inhibits sulphanilamide action, namely peptone. As this peculiar antagonist offers some interesting outlooks on the mechanism of sulphanilamide action, we will consider the facts as far as we studied them more in detail.

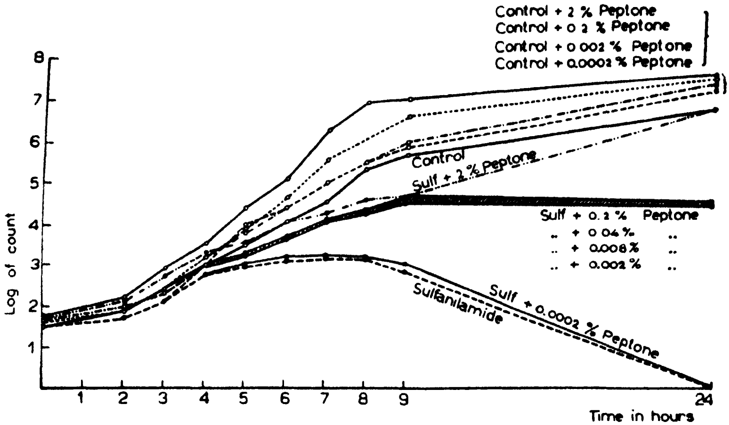


Fig. 7. Influence of various concentrations of peptone (British Drug Houses) on the action of 0.1 % sulphanilamide on Bacterium coli in a synthetic medium.

According to Fig. 7, peptone shows a marked inhibition of sulphanilamide action, but it is incomplete. Besides, it is remarkably constant in concentrations between 0.002 and 0.2 %,

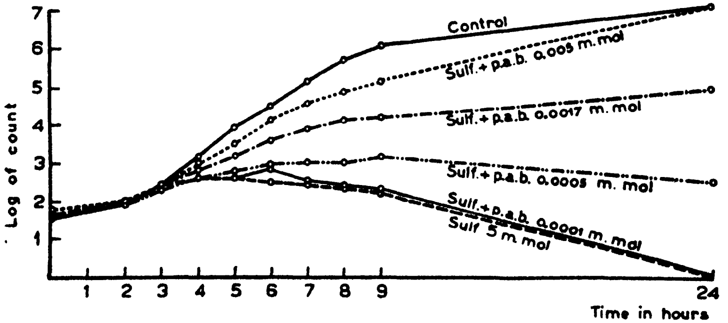


Fig. 8. Influence of various concentrations of p-aminobenzoic acid on the action of 0.1 % sulphanilamide on Bact. coli in a synthetic medium.

whilst the counteraction of p.a.b.a. varies with the concentration as read from Fig. 8.

Only with as much as 2 % peptone an additional and more complete inhibition is obtained. These facts seem to trend towards the supposition that perhaps two inhibitory potencies are present in peptone.

A colorimetric "determination" of p.a.b.a. in 10 proc. peptone solution shows a colour corresponding with 0.05 m. mol. p.l. of p.a.b.a. Of course the actual concentration will be much lower as most of the colour is due to other constituents. Even after hydrolysis of the peptone by means of sulphuric acid in an autoclave no increase of the colour reaction was observed (in the case of the peptone we used). Thus peptone inhibits sulphanilamide at least 150 times more strongly than would correspond with its maximal p.a.b.a. content; only the additional inhibition of 2 % peptone might be due to p.a.b.a. (see Fig. 7).

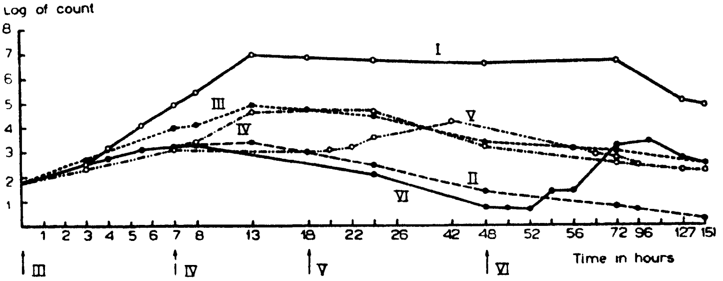


Fig. 9. Addition of peptone after various periods. Bact. coli, synthetic medium, 0.1 % sulphanilamide, 0.02 % peptone added after 0, 7, 18 and 48 hours.

Another argument stresses the difference of peptone and p.a.b.a. counteraction, viz., that in some media containing peptone, sulphanilamide has its full activity whilst p.a.b.a. is still counteracting in very low concentrations.

Thus the inhibiting action of peptone cannot possibly be due to p.a.b.a.

Fig. 9 shows the effect of peptone addition to cultures of Bact. coli in a synthetic medium containing sulphanilamide.

From Curve III when compared with Curve II it can be read that on adding peptone the peculiar maximum discussed on page 12 is increased, but afterwards the bacteria still die off and the curve declines parallel to Curve II (i.e., the culture with sulphanilamide and without peptone).

On adding peptone after a period of undisturbed sulphanilamide action, a "second" maximum appears (Curve IV, V and VI), but again the bacteria finally die off in much the same way as in the culture with mere sulphanilamide.

From Fig. 10 the result is, that the influence of peptone in increasing the level of the maximum is in some degree independent of the concentration of peptone. Another addition after some time fails to repeat the effect. So perhaps it is preferable in the case of Curves V and VI (Fig. 9) not to speak of a "second" but better of a "delayed" maximum. It must be kept in mind that the addition of p.a.b.a. in sufficient concentration, after some period of undisturbed sulphanilamide action, would immediately produce a complete recovery of the bacteria and multiplication to the level of the control by complete absence of a maximum. So we may conclude: Peptone in not too high a concentration prolongs the lag-time

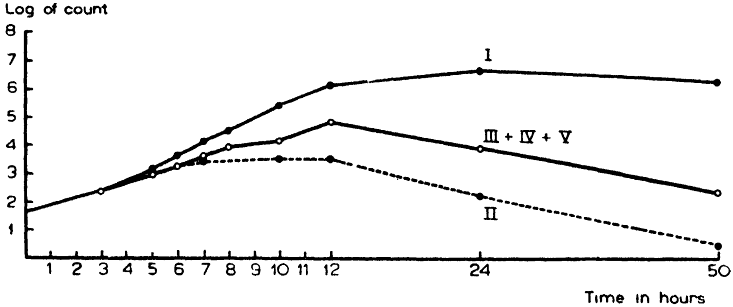


Fig. 10. Influence of peptone on sulphanilamide action. Bact. coli, synthetic medium, 0.1 % sulphanilamide.

I control.
II with sulphanilamide.
III id. + 0.01 % peptone after 5 h
IV id. + 0.02 % peptone after 5 h
V id. + 0.01 % peptone after 5 h + 0.01 % peptone after 8 h.

of sulphanilamide effect (i.e., increases the maximum in the growth curve), thus having but a limited period of "counteraction". P.a.b.a. does nothing of the kind.

The leading feature is that peptone only seems able to give the bacteria a temporary "leg up" by permitting growth to a limited level, but is, apart from very high concentrations, unable to withdraw the bacteria definitely from the injurious effect of sulphanilamide action. The action of p.a.b.a., on the contrary, is permanent and, in an appropriate concentration, absolute.

As the inhibition of sulphanilamide action by peptone is incomplete, temporary (prolonged lag-time of sulphanilamide effect) and independent, within wide limits of the peptone concentration, it is evident that the mechanism of peptone antagonism differs widely from that of p.a.b.a. So widely that one has to discriminate for sulphanilamide-poisoned bacteria between the antidote p.a.b.a. and the tonic peptone.

Some consequences from the above facts for the mechanism of sulphanilamide action will be discussed below.

Calcium.

Very early in our work (unpublished) we found Ca-ions to antagonize sulphanilamide action. A typical experiment with streptococci in blood broth is given in Fig. 11.

With Bact. coli in a synthetic medium the antagonism was also found. Ca″ is able to antagonize sulphanilamide, even when added some hours after the sulphanilamide. It seems to prevent the dying off of the bacteria, i.e., to act in the third phase. Ba″, Sr″ and Mg″ are quite inactive. Though interesting, the antagonism Ca″-sulphanilamide probably has no bearing on the real mechanism of sulphanilamide action and must be probably explained by changes in cell permeability.

Ca″ might inhibit the diffusing out of the cell of some products involved in the reactions with which sulphanilamide interferes. This substance might be p.a.b.a. or a peptone-like substance.

Cysteine.

Cysteine partially counteracts sulphanilamide (with strepto-

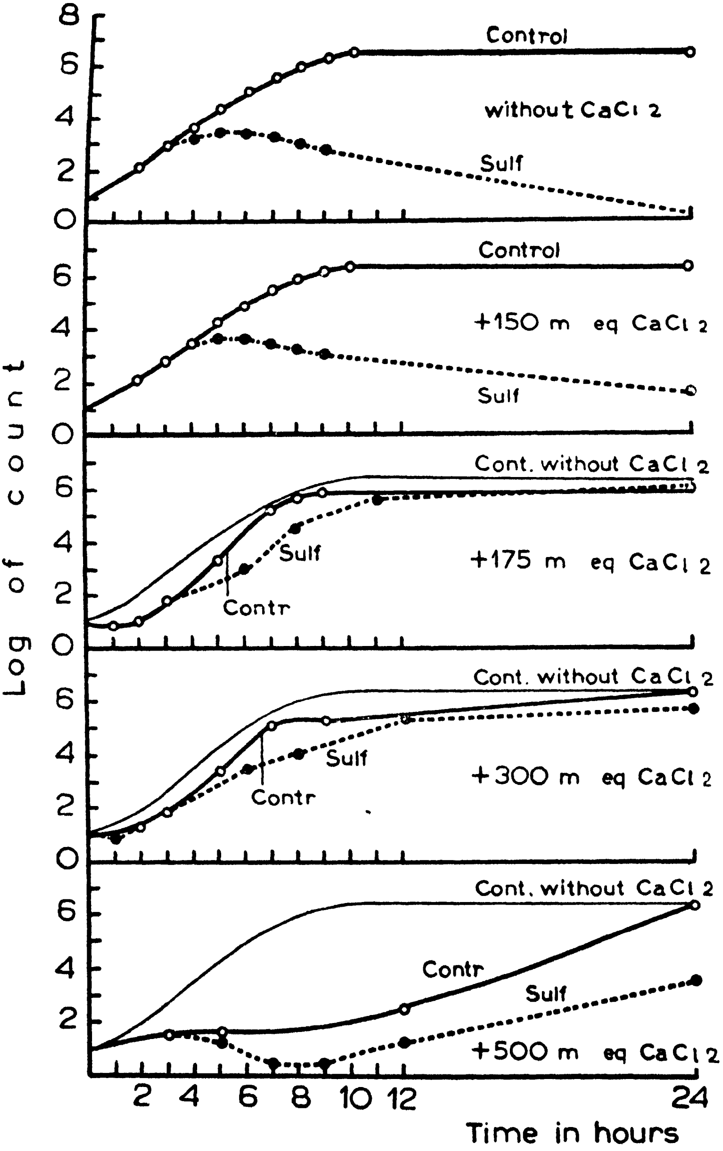


Fig. 11. Influence of $CaCl_2$ on sulphanilamide action. Streptococci, 50 % horse-blood broth.

cocci) according to our experiments (JULIUS and WINKLER, 1942) and seems to act in the third phase. We have no explanation to offer, though we might refer to p. 42.

Methionine.

BLISS and LONG (1941) have shown that methionine, in itself an antiseptic, can antagonize sulphanilamide action. For streptococci in blood broth or Bact. coli in a synthetic medium, we could not confirm this statement, but this can easily be due to experimental conditions (compare HARRIS and KOHN, 1941).

Nicotinamide.

Nicotinic acid amide was said to antagonize sulphanilamide. We could not confirm this fact for staphylococci.

Adenine.

Adenine in excessive concentration seems to antagonize sulphanilamide action in vivo (MARTIN and FISHER, 1942). In vitro with streptococci in blood broth we did not observe any antagonism.

p-Aminobenzoyl-l-glutamic acid.

AUHAGEN (1943) brought evidence that p-aminobenzoyl-l-glutamic acid (p.a.b.g.a.) was ten times as active as a growth substance for *Streptobacterium plantarum* as compared with p.a.b.a. To the synthetic medium on which *Streptobacterium plantarum* developed, AUHAGEN added some sulphanilamide; therefore it is not clear if he did study the action of p.a.b.g.a. as a growth factor or as a sulphanilamide-antagonist, as meant in the heading of his paper. We found p.a.b.g.a. much less active than p.a.b.a. in antagonizing sulphanilamide in the case of *Bact. coli* in a synthetic medium of streptococci in blood broth. VELDSTRA and HAVINGA (see page 116) corroborated our finding as they found p.a.b.g.a. inactive in antagonizing sulphanilamide in vitro (in vivo, probably by splitting off p.a.b.a., it had the same activity as p.a.b.a. itself). The same lack of antagonistic power we observed in the case of a strain of ***Streptobact. plantarum***[1] in glucose broth. It would of course be very interesting to study the activity of p.a.b.g.a. as a growth factor in comparison with p.a.b.a. on the same strain of ***Streptobact. plantarum***, but various ingredients for the complicated synthetic medium were lacking during the war.

The question must be considered however, as one of primary importance for the conception of sulphanilamide action as it gives rise to the following considerations:

If p.a.b.g.a. is more active as a growth factor than is p.a.b.a., (AUHAGEN, 1943) but not an active sulphanilamide antagonist (considering our experiments), this would be in serious contradiction with the theory that sulphanilamide acts by displacing p.a.b.a. IVANOVICS (1944), though admitting too readily that p.a.b.g.a. is active as a growth factor but not as an antagonist, fails to draw this far-reaching consequence!

If, on the other hand, AUHAGEN studied the antagonism, we must state that we have not been able to confirm the increased activity of p.a.b.g.a. as compared with p.a.b.a., which ought to be apparent. This activity could be limited to a special strain or a special medium. It might then become important to know if other p-aminobenzoyl-amino acids might show an increased activity for other strains and other bacterial species, thus throwing a light on the mechanism of p.a.b.a. action.

Further experiments will have to decide these questions.

β. Activators.

Many influences will alter the velocity of biochemical reactions. The process which is involved by sulphanilamide action also will respond to any change of condition. Together with antagonizing influences, activating ones also must be expected. In an early period of our study of sulphanilamide action, an influence of sulphanilamide on cell permeability was supposed, and we studied the influence on sulphanilamide action of various factors, usually increasing or reducing the permeability of cell membrane. The inhibition of sulphanilamide by Ca-ions, presumably due to a reduction of permeability, has already been mentioned under "Antagonists".

---

[1] We thank Prof. KLUYVER (Delft), for kindly supplying the strain of *Streptobact. plantarum* (Orla JENSEN) that probably is identical with the strain used in the Heidelberg laboratories. Prof. KUHN unfortunately declared himself unable to send us his special strain.

Some factors enhancing sulphanilamide action will be mentioned here.

Rise of temperature.

It was already known (WHITE, 1939) that sulphanilamide was more active at higher temperatures (keeping within the range of temperature permitting bacterial growth) showing a relatively high temperature coefficient.

At higher temperatures permeability is as a rule increased; there are, however, many other possibilities to explain the influence of temperature on sulphanilamide action.

Decreasing acidity of the medium.

In accordance with other investigators we found that sulphanilamide is more active at higher pH values, between pH 6 to 8. Here again the question of permeability might be involved.

Organic solvents.

The biochemical action of alcohols, ether, chloroform, and other lipoid solvents is generally supposed to be due to their lipoid solving properties, by which an increased permeability is obtained.

With small concentrations of chloroform (0.3 proc.) we obtained an activation of sulphanilamide action for Bact. coli in aqueous peptone (unpublished).

With normal alcohols the action of sulphanilamide on streptococci in 50 proc. (sheep) blood broth was enhanced. Equal activity was obtained with 4 proc. ethyl alcohol, 1.6 proc. propyl alcohol and 0.6 proc. butyl alcohol. Such a decrease of active concentration might — according to TRAUBE's rule — again suggest an influence on permeability.

We do not think the enhancing action of the substances mentioned very essential, as each of them in itself has antiseptic *properties in higher concentration*, and as such a potentiation of drugs is not unusual in pharmacology. Nevertheless the phenomenon will have to be kept in mind.

Horse blood.

WOLFF and JULIUS (1939) observed that sulphanilamide was much more active on streptococci in media containing 50 proc. horse blood than in the same media containing an equal amount of sheep blood. WINKLER and JULIUS (1942), in an extensive study on this subject, showed that the red cells of the horse blood contain a substance or substances activating sulphanilamide even when greatly diluted (up to 0.5 proc. red cells). In some media no sulphanilamide action was obtained without the activator, in other media sulphanilamide action was merely enhanced. The substance was contained in the hemoglobin fraction and presumably of protein nature or adsorbed to the protein.

The substance was activating sulphanilamide against streptococci, staphylococci, and *Bact. coli.* Other bacteria were not tested. The substance is not present in human blood or in the red cells of sheep, rabbit, or mouse. The substance was supposed to enhance sulphanilamide action by an improvement of penetration of sulphanilamide into, or adsorption to the cocci. Though several attempts at isolation of the substances involved have failed as yet, we think the existence of this activator important, as the observed facts might contribute to our knowledge of the mechanism of sulphanilamide action and even be of therapeutic value.

Summary.

1. The antagonism between sulphanilamide and p-aminobenzoic acid is discussed and though it is admitted that p.a.b.a. probably counteracts by displacing sulphanilamide, it is held that the explanation of sulphanilamide action by a displacement of p.a.b.a. has not yet been proved.

2. It is shown that the sulphanilamide antagonizing action of peptone is not due to p.a.b.a., that peptone inhibition is incomplete, temporary (only delaying the effect of sulphanil-

amide action) and within wide ranges independent of the concentration of the peptone added. It is concluded that the mechanism of peptone action differs fundamentally from that of p.a.b.a.

3. It is shown that Ca-ions are able to inhibit sulphanilamide action completely.

4. The antagonistic properties of cysteine, methionine, nicotinamide and adenine are discussed.

5. The growth promoting and antagonistic properties of p-aminobenzoylglutamic acid and their bearing upon the mechanism of sulphanilamide action are discussed.

6. The enhancing influence of rising temperature, OH-ions, chloroform, and normal alcohols on sulphanilamide action is discussed.

7. It is shown that the red cells of the horse contain a substance activating sulphanilamide even in great dilution. The substance is present in the hemoglobin fraction and presumably of protein nature or adsorbed to the protein.

e. Discussion on the action of sulphanilamide.

a. The origin of the maximum in growth curves with sulphanilamide.

Above (page 12) the peculiar maximum in the growth-curve of cultures containing sulphanilamide was mentioned as one of the chief characteristics of the action of sulphanilamide. Several explanations are possible. We will discuss their probability here.

1. One might suppose the velocity of permeation or of an adsorption process to be the determining factors to which the delayed action is due. The great velocity of the adsorption of sulphanilamide derivatives by bacteria on the one hand, the occurrence on the other hand of instantaneous growth when bacteria are taken out of cultures containing sulphanilamide and inoculated in media free from sulphanilamide, do not sustain these views.

2. The action of sulphanilamide might be instantaneous but the effect might be delayed by some antagonistic potency present in the inoculum, or in the medium or by some reserve of substances in the bacteria, the production of which is in hibited by sulphanilamide, permitting some multiplication.

It is evident that such an antagonist or reserve substance can not be p.a.b.a. (Woods, 1940) or the like, as, in that case, the maximum should disappear at higher sulphanilamide concentrations, or increase by addition of p.a.b.a., neither of which is the case (see figs. 1 and 17).

The supposed antagonist or reserve material might be some compound analogous to one of the constituents of peptone. This seems a reasonable supposition, as peptone increases the height of the maximum, thereby delaying the effect of sulphonamide action (see page 28).

This view indeed is corroborated by the experiment presented in Fig. 12.

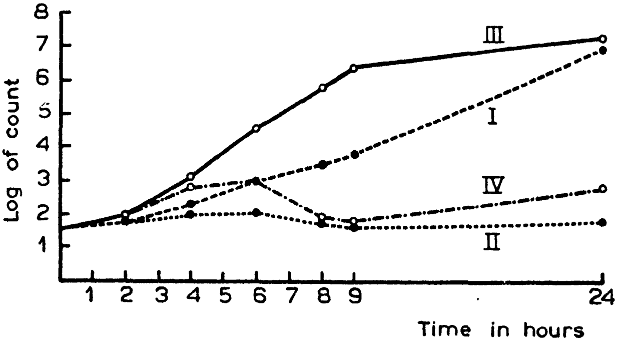


Fig. 12. Influence of an "optimal" mixture of amino acids on sulphanilamide action. Bact. coli, synthetic medium.
I Control.
II With sulphanilamide.
III With amino acids.
IV With amino acids and sulphanilamide.

Under the special conditions realized, the maximum in the culture with sulphanilamide (Curve II) is absent, in itself an extraordinary observation, which will be discussed below. The addition of a mixture of amino acids — peptone would have had the same effect — at once introduces the well known phenomenon of the elevation in the curve (IV).

Some experiments seem to show that the height of the maximum decreases when washed inocula are used. On the other hand, as it has been impossible to suppress the maximum altogether by washing and the effect of washing is inconstant, the substances involved are probably present in the bacteria and not very readily given off to the medium.

So we think that the only explanation, covering all the facts, is an instantaneous action of sulphanilamide, interfering with some part of bacterial anabolism, delayed as a rule by the presence in inoculum or medium of some reserve-substance which is not p.a.b.a. or a like substance, but acts by the same mechanism as the factors in peptone. This suggests, by the way, that the term antagonistic, as it is used till now for the effect of peptone, can be better reserved for true antagonists like p.a.b.a., whereas peptone is better defined as a "tonic" only.

The explanation of the maximum by the presence of antagonistic substances does not invalidate our view that the maximum is one of the characteristic features of sulphanilamide action. The possibility of such an antagonism remains the very characteristic feature.

β. The action of sulphanilamide in cultures with a long lag-time (of growth).

Another fact regarding the maximum calls for our attention. Some allusions to it have already been made; reviewing a great number of growth-curves of cultures containing sulphanilamide, including various bacteria in different media, we observed that the maximum was always present, except in media showing an unusually long lag-time before growth set in. In those cases the maximum was absent or very little accentuated, the bacteria dying off from the beginning, and the curve showing the second and third phase only (see page 11), as for instance in Fig. 13, Curve II.

The experiment in Fig. 13 gives direct evidence that it is the long lag-time of growth which causes the absence of the maximum, because the addition of sulphanilamide at the end of the lag-period results in the usual curve with a maximum (Curve III). The same was observed with various bacteria in several media. In line with our supposition that the maximum is due to peptone-like substances in the inoculum or in the medium, this would mean that in the cultures with a long lag-time of growth these substances are absent. A short lag-time is known to occur with: a. optimal media, b. large inocula, or c. after transplantation during the logarithmic phase of the mother culture, that is, in cases in which one might suppose a sufficient supply of those substances to be present (a and b), or to be synthesized with great velocity (c). A long lag-time of growth is observed with small inocula and in poor media, the lag-time probably being necessary to the synthesis of substances required for growth and multiplication.

As sulphanilamide acts without delay in cultures showing a long lag-period, whilst the effect goes through a maximum when the lag-period is short or when sulphanilamide is added at the end of the (long) lag-time, this means — considering the maximum to be due to the presence of antagonists of the peptone type — that these antagonists are produced during the (long) lag-period, and also that their production is impossible when sulphanilamide is present.

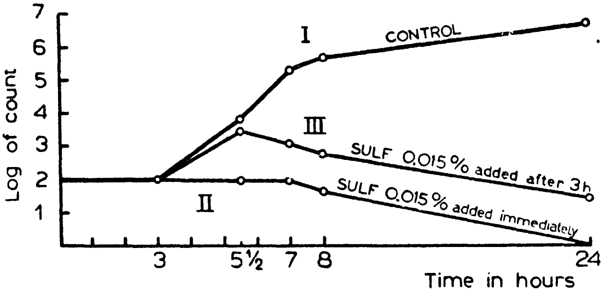


Fig. 13. Influence of sulphanilamide in cultures with a long lag-time (of growth). Pneumococci, horse-blood broth. 0.015 % sulphanilamide.

Quite apart from this important conclusion the above experiments show that sulphanilamide can act before multiplication sets in.

Our view (see page 15), that cell division or growth is involved in the mechanism of sulphanilamide action seems to be invalidated by this last experiment. But, in fact, in cultures with a long lag-time of growth sulphanilamide is active only when the medium contains substances permitting growth in the control culture. This is the essential feature that can be read from Fig. 6, where $(NH_4)_2SO_4$ was the only source of nitrogen.

On the other hand, our former view that some cell divisions were necessary to sulphanilamide action cannot be upheld. Sulphanilamide interferes with the anabolism that precedes multiplication or with the first stages of multiplication itself. If the possibility for anabolism fails, even the highest concentration of sulphanilamide is without influence on the bacteria. Of course, the aforesaid is in full accordance with our supposition about the influence of sulphanilamide on bacterial N-metabolism.

γ. *The mechanism of peptone action.*

To illustrate the complicated relationships involved in the antagonizing properties of peptone and the maximum in the growth curve of cultures with sulphanilamide, we should like to make use of a diagram. We want to stress, however, that this diagram is only meant as an illustration of the limited antagonism of peptone. It should not be accepted as a theory of sulphanilamide action, which most definitely it is not, though of course some of our ideas and observations on sulphanilamide action are included.

Sulphanilamide is inactive against resting bacteria or large inocula, whereas the activity is marked when small inocula are used (in a suitable medium). In the latter case the bacteria start growing, but in the presence of sulphanilamide the produced bacteria are of an inferior quality and die off (third phase, (see page 11). Let the inferior quality be due to a partial or total lack of some substance (enzyme or catalyst) necessary to bacterial maintenance as already suggested on page 21. (Instead of a real lack of this substance — to be called E in diagram (1) — one might of course suppose an increased consumption or need of the substance E by growing bacteria as compared with resting bacteria).

This substance E is produced during normal growth but the production is inhibited when sulphanilamide is present. Hence the survival of very large inocula in spite of sulphanilamide (see Fig. 5, Curve VI) or resting bacteria (which were of course grown without sulphanilamide), as both possess a normal supply or reserve of E.

As the synthesis of E will be the result of some series of biochemical reactions, one might represent this by

$$A \mathrel{-\!\!\mid\!\!\rightarrow} B \rightarrow C \rightarrow D \rightarrow E \qquad (1)$$

The black line between A and B represents the step in this chain which is blocked by sulphanilamide; p.a.b.a. here can displace sulphanilamide and re-establish the blocked mechanism.

As the O_2-consumption of bacteria grown in sulphanilamide is equal to that of control cultures, it is improbable that E should be a substance (ferment, co-ferment) necessary to respiration. As the lack of E, however, not only inhibits further growth but definitely results in a dying off of the bacteria, E will be responsible for some part of the metabolism necessary for survival, probably not involving protein synthesis (as bacteria in media without N-supply survive several days). Such a part of metabolism might consist in the use of respiratory energy for survival (keeping up of bacterial structures, membrane potentials and the like).

It is evident that the substance indicated as E cannot be p.a.b.a. itself. In that case a certain amount of p.a.b.a. should permit growth and multiplication in the presence of sulphanilamide, quite independent of the concentration of the latter substance, which is in direct disagreement with the constant ratio $C_{\text{sulphanilamide}} : C_{\text{p.a.b.a.}}$ found for this antagonism.

Peptone-like substances, responsible for the maximum, are present in most bacteria (as a maximum also appears in synthetic media, or with washed inocula). On the other hand, these substances are produced during the lag-time preceding growth, and this production is inhibited by sulphanilamide.

This might be represented by:

$$(A \mathrel{-\!\!\mid\!\!\rightarrow} B \rightarrow C \rightarrow P) \qquad (2),$$

P representing the peptone-like substances.

It can be asked now if the antagonizing substances in peptone, i.e., the substances causing the maximum might be identified with E, that is, with the final product of the reaction blocked by sulphanilamide.

Some further reflections show that this cannot be the case. For, if this were true, the inhibition of sulphanilamide action would increase proportionally with the amount of peptone added above a certain minimum, until a complete inhibition was obtained. According to Fig. 7 and 10 this is not the case.

As the substance P cannot completely inhibit sulphanilamide action, some other factor is still inhibiting the production of E, e.g.:

$$\begin{tikzcd} A \arrow[r, "|" marking, dashed] & B \arrow[r] & C \arrow[r] & \left.\begin{array}{c} P \\ + \\ X \end{array}\right\} \arrow[r] & E \end{tikzcd}$$

The maximum in the growth curve is thus explained by the present stock or supply of P and X; addition of peptone increases the maximum, but does not produce a complete inhibition of sulphanilamide action, as the production of E is still limited by lack of X.

In synthetic media the substance X has to be produced by the bacteria. This production evidently is reduced also by the presence of sulphanilamide, otherwise the supply could not be limited and again peptone would be a complete antagonist. So our diagram reads:

$$\begin{tikzcd} & & C \arrow[r] & P & & \\ A \arrow[r, "|" marking, dashed] & B \arrow[ur, dashed] \arrow[dr, dashed] & & & nP + X \arrow[r] & E \\ & & O \arrow[r] & X & & \end{tikzcd}$$

We write nP to account for the fact that P and X are not present in equivalent amounts, so that X becomes a limiting factor only after the addition of an extra supply of P.

HARRIS and KOHN (1941) proposed an analogous hypothesis for sulphanilamide action.

The only thing we want to illustrate with this diagram is that

1. The maximum is due to a reserve of substances (P and X) in the bacteria.

2. The addition of peptone increases the maximum, practically independent of the peptone concentration, but does not

produce complete inhibition of the sulphanilamide action, the increased maximum still being limited by the lack of X.

Every feature of the sulphanilamide antagonizing action of peptone is thus illustrated in the diagram.

We wish to add that the substance X cannot be p.a.b.a., as this does not increase the maximum. The additional inhibition of sulphanilamide action by a very high concentration of peptone might be due to the substance X. A study of substances completing the inhibition of (our!) peptone will be important.

On page 36, an experiment was presented in which a mixture of amino acids produced a maximum in the growth curve of a sulphanilamide-containing culture, whilst it was absent in the corresponding culture without amino acids. Considering our present views about the maximum, the question arises whether our former conclusion, that sulphanilamide does not interfere with bacterial amino acid synthesis, can still be upheld.

The addition of an optimal mixture of amino acids does not inhibit sulphanilamide action, so in a general way the conclusion seems valid; on the other hand the added amino acids at least permit some growth in the presence of sulphanilamide. Some influence on amino acid synthesis has to be admitted.

Referring to our diagram, sulphanilamide interferes indeed with amino acid synthesis (substance P), but the more important feature of sulphanilamide action seems to be contained in the inhibition of the production of X (or Y or Z), which even after the addition of amino acids acts as a limiting factor for bacterial growth (and for the production of E).

Indeed one of the most important features of the above diagram (or rather of the observation of the partial inhibition by peptone)seems to be the realization that still other antagonists (substance X) which, combined with peptone, should completely inhibit sulphanilamide action must be in existence. By finding these substances not only the mechanism of sulphanilamide action, but also some parts of bacterial anabolism might be better understood.

Concluding, we think that the antagonizing action of peptone is a worthy counterpart to that of p.a.b.a. It seems to contribute in several ways to the conception of the mechanism of sulphanilamide action, especially as regards the maximum in the growth curve, which is one of the chief characteristics of the peculiar action of sulphanilamide and which cannot be explained from the counteraction by p.a.b.a.

Summary.

1. The maximum in growth curves with sulphanilamide is shown to be due to a reserve in the bacteria or in the medium of peptone-like substances, delaying the effect of sulphanilamide action.
2. In cultures showing a long lag-time before multiplication sets in, the effect of sulphanilamide action is not delayed, the maximum is absent, but reappears when sulphanilamide is added at the end of the lag-period only. The substances responsible for the maximum are produced during the lag-period and their production is stopped by sulphanilamide.
3. A diagram illustrating the mechanism of peptone action and some of the problems of the maximum are discussed.

f. The mechanism of the action of sulphanilamide derivatives.

Since the discovery of sulphanilamide, an innumerable number of substituted sulphanilamides have been synthesized. Some of them were inactive, some equalled sulphanilamide, a few were much more potent than sulphanilamide, thus extending our chemotherapeutic arsenal with substances, active against bacterial infections which could not be cured with sulphanilamide, or showing an enhanced action against infections, in the fighting of which sulphanilamide was already in use.

The mechanism of the action of the latter kind of derivatives as compared with that of sulphanilamide will be discussed here. A study of the mechanism in vitro will be followed by a discussion of the possibilities in vivo.

a. *In vitro.*

It soon became evident that all the drugs of the sulphanilamide group actually showed the characteristic features of sulphanilamide action, mentioned before:

a. The activity of the derivatives of sulphanilamide in vitro is obtained only with small inocula and actively multiplying bacteria.

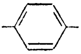
b. The addition of blood seems to enhance the sulphonamide effect.

c. The typical form of growth curve is obtained with every drug of this group.

d. The action of every derivative is counteracted by very small concentrations of p.a.b.a., and, last but not least:

e. bacteria resistant towards sulphanilamide are equally resistant towards the derivatives.

So it seems that, at least in vitro, the mechanism of action on bacteria of all the derivatives in this group is the same.

This is so evident, that we did not hesitate (Winkler and Julius, 1944), to state that a drug like p-aminomethylbenzenesulphonamide (Marfanil, NH_2CH_2--SO_2NH_2) could not possibly belong to the sulphanilamide group, as most of the above-mentioned features (especially those under c. and d.) were absent. This view was corroborated by the fact, that bacteria resistant towards Marfanil were not resistant towards sulphanilamide; the opposite also holds true (see also Jensen, Schmith and Brandt, 1942).

It is beyond doubt, that some of the derivatives of sulphanilamide (sulphapyridine, sulphathiazole, a.o.) are more potent drugs (at least in vivo) than sulphanilamide; the question arose whether also in vitro differences in activity could be found. A quantitative analysis of the conditions in vitro showed indeed (see below) a distinct quantitative difference in the activity of sulphanilamide as compared with several of its derivatives.

The mechanism of action being the same, a higher activity (in vitro) of the derivatives as compared with the mother substance, can only mean the same activity in lower concentrations. Indeed, in growth experiments, the only differences between sulphanilamide and e.g., sulphamethylthiazole appears to be a difference in concentration. (Fig. 14 and 15).

The question then arises: why smaller amounts of sulphamethylthiazole are equally active as sulphanilamide, the mechanism being the same.

An adsorption process might explain this. As the activity will be due to "adsorbed" molecules only, the adsorbed quantity of both substances might be equal at very different concentrations.

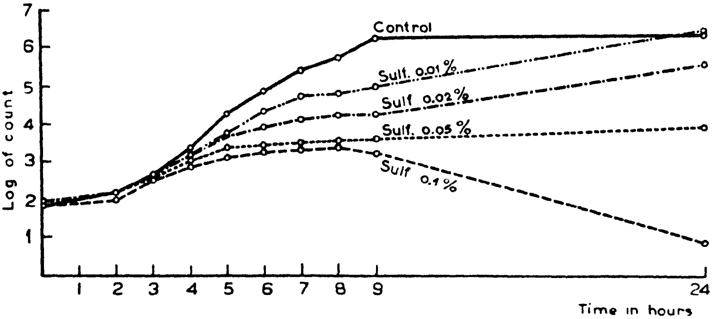


Fig. 14. Growth curves of Bact. coli in a synthetic medium with various concentrations of sulphanilamide (0.1 % = 6 m.mol.).

To test this hypothesis we studied how far the activity of thirteen sulphanilamide derivatives was related to their adsorption into the bacteria (Bact. coli) (JULIUS and WINKLER, 1943, 1945).

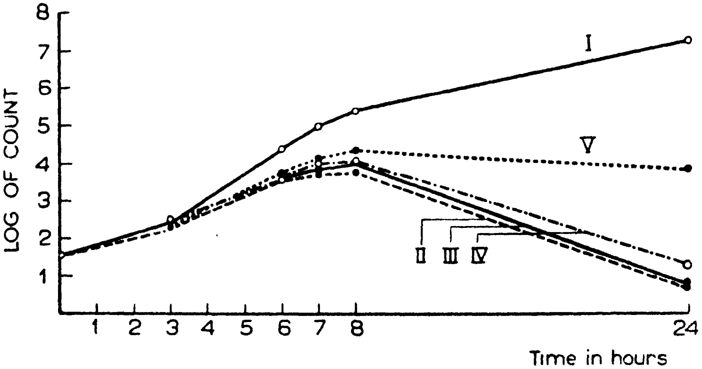


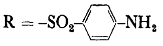
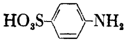
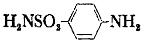
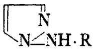
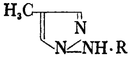
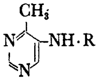
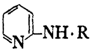
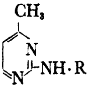
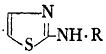
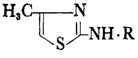
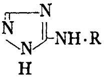
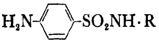
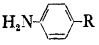
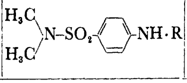
Fig. 15. Growth curves of Bact. coli with various concentrations of sulphamethylthiazole. Curve II to V 0.6, 0.2, 0.07, 0.03 m.mol. sulphamethylthiazole; minimal active concentration 0.07 m.mol.

The minimal active concentration of each drug was taken from a series of growth curves with various concentrations of each drug and arbitrarily defined as the concentration, just able to reduce the log

TABLE I

ALL CONCENTRATIONS ARE GIVEN IN MILLIMOLES PER LITRE.

Column a: Minimal concentration of the drug which is active, i.e., reduces the number of viable bacteria below 10 per standard droplet, i.e., 350 per ml, in 24 hours.
Column b: Activity coefficient with regard to sulphanilamide.
Column c: Adsorbed quantity x/m, in millimoles, per litre of bacteria.
Column d: Smallest concentration of p.aminobenzoic acid, which is completely inhibiting the effect of them inimal active concentration of the drug.
Column e: a/d = Ratio between active concentrations of p-aminobenzoic acid and sulphanilamide or related drugs.
Column f: b × e = 6/a × a/d = 6/d = Constant.
□ = No adsorption within the experimental error.

Nomenclature	Structure 	a	b	c	d	e	f
Sulphanilic acid		116	0.05	□	0.005	23000	∼1200
Sulphanilamide		6	1	□	0.005	1200	1200
2-Sulphanilylamino-pyrazole		4	1.5	□	0.005	800	1200
2-Sulphanilylamino-4-methylpyrazole		4	1.5	□	0.005	800	1200
5-Sulphanilylamino-4-methylpyrimidine		0.35	17	0.11	0.005	70	1200
Sulphapyridine		0.20	30	0.08	0.005	40	1200
2-Sulphanilylamino-4-methylpyrimidine		0.15	40	0.07	0.005	30	1200
Sulphathiazole		0.08	75	0.12	0.005	16	1200
Sulphamethylthiazole		0.07	85	0.15	0.005	14	1200
Sulphatriazole		inactive >4		□			
Di-sulphanilamide		inactive >4		□			
Diamino-diphenyl-sulphone		0.8	7.5	4	0.005	160	1200
Uliron		inactive >0.028 (saturated)		strongly adsorbed			

of count within 24 hours below 1 (~10 bacteria per unit of volume — in our technique a droplet of 28 mg — that is 350 per ml).

The adsorption was determined by adding various concentrations of the special drug to concentrated suspensions of Bact. coli and, after centrifuging, estimating the concentration of the drug in the supernate.

The minimal active concentrations (m.a.c.) are collected in Table I, Column a, whereas the relative activity with regard to sulphanilamide can be found in Column b. Sulphapyridine is 30 times, sulphathiazole 75 times more active than sulphanilamide, etc. The adsorption, as estimated in our experiment, seems to be a vital feature, as heating of the bacteria up to 60° C. destroys their capacity to adsorb sulphonamides. Neither are these drugs adsorbed (within the experimental error) to erythrocytes or liver cells.

In Fig. 16 the log of the adsorbed quantity per unit of bacteria (log x/m) is plotted against the log of the equilibrium concentration in millimoles per litre.

Apart from p.a.b.a., which is adsorbed better than any of the sulphonamides, the latter are ordered in a series of decreasing degree of adsorption:
Sulphamethylthiazole > sulphathiazole > 5-sulphanilylamino-4-methylpyrimidine > sulphapyridine > 2-sulphanilylamino-4-methylpyrimidine > sulphanilamide.

The sulphapyrazoles, sulphanilamide, and sulphanilic acid were not adsorbed within the experimental error.

The order of sequence of increasing adsorption is the same as the order of sequence of increasing activity. Considering the adsorption values obtained, which have a considerable experimental error (about 2 %), as real, a quantitative relation between activity and adsorption seems indeed to exist.

Table I, Column c, shows the adsorbed quantities, intrapolated from Fig. 16, in relation to the m.a.c. They are nearly equal, i.e., about 0.1 millimoles (per litre of bacterial substance).

This may also hold for sulphanilamide and the pyrazoles, but escapes experimental corroboration as the adsorption is within the experimental error. The dotted curves for these substances were drawn, supposing that for these drugs the adsorbed quantity at m.a.c. might also be 0.1 millimoles.

Only two out of the thirteen substances seemed to be in conflict with the described correlation. A non-specific adsorption at inactive centres might explain the excessive adsorption of diaminodiphenylsulphone, whereas some sterical hindrance might explain the inactivity of Uliron against Bact. coli in our experiments.

However, from the (quantitative?) correlation between activity and degree of adsorption, one might conclude generally

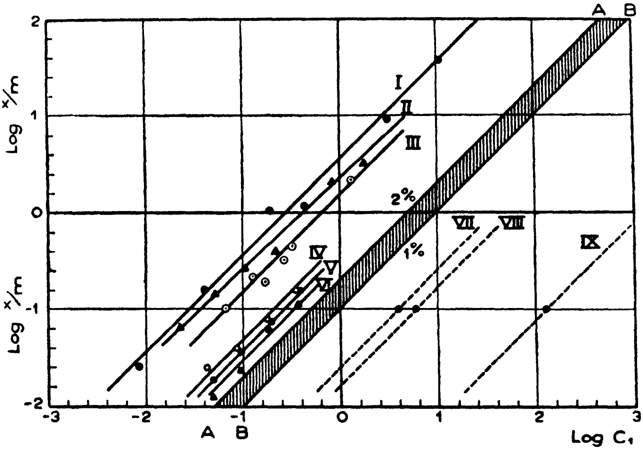


Fig. 16. Adsorption of various sulphonamides by Bact. coli.
log x/m = adsorbed quantity, in millimoles, per litre of bacteria.
log C_1 = equilibrium concentration in millimoles per litre.
I p.aminobenzoic acid.
II sulphamethylthiazole.
III sulphathiazole.
IV 2-sulphanilylamino-4-methylpyrimidine.
V sulphapyridine.
VI 5-sulphanilylamino-4-methylpyrimidine.
A-A limit of measurableness for an experimental error of 2 %.
B-B limit of measurableness or an experimental error of 1 %.
VII supposed curve for both the sulphanilylamino-pyrazoles.
VIII supposed curve for sulphanilamide.
IX supposed curve for sulphanilic acid.

that the better action of the studied sulphanilamide derivatives in vitro is due to a better adsorption.

Apart from the above adsorption experiments, which one might claim to be accidental or due to non-specific adsorption, another argument corroborates our view.

Quite apart from the relative activity or from the absolute value of the m.a.c. of each drug, the concentration of p.a.b.a. counteracting the m.a.c. (Fig. 8 and 17) is equal for each compound (Column d).

Analogous results were obtained by Rose and Fox (1942), Ivanovics (1944) and others. From growth curves with streptococci in blood broth, and staphylococci in a semi-synthetic medium, the same relation could be established extinctiometrically. This means that a constant amount of p.a.b.a. molecules is necessary to displace the adsorbed molecules of each drug, from which follows that for every drug the adsorbed quantity must be the same. So independently we arrive at our former conclusion that the different activity of the various sulphanilamide derivatives is due to differences in the degree of adsorption.

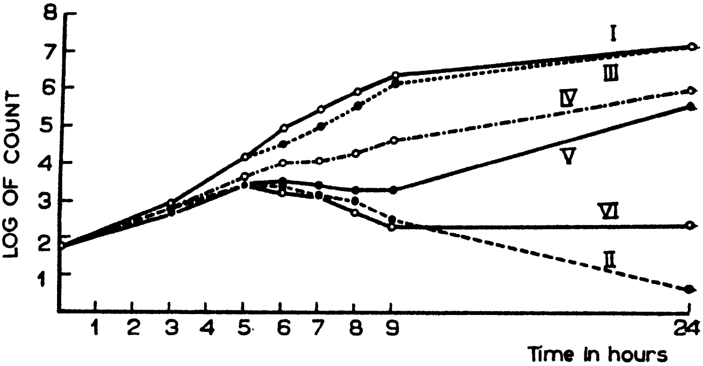


Fig. 17. The inhibition of the activity of sulphapyridine by p.aminobenzoic acid (Bact. coli).

I control.
II with 0.15 m.mol. of sulphapyridine.
III, IV, V, VI with 0.15 m.mol. of sulphapyridine and 0.0001; 0.0005; 0.0017; 0.005 m.mol. of p.aminobenzoic acid.
Complete inhibition with 0.005 m.mol. of p.a.b.a. (Curve VI).

In relation to the above experiments and with a bearing on the following discussion on relations in vivo, we reproduce in

Table II the m.a.c. for various compounds as derived from growth curves and extinctiometric observations for some kinds of bacteria.

With Bact. coli there is an enormous difference between sulphanilamide and sulphapyridine. Apart from this difference the sulphapyrimidines and sulphapyridine generally are 2–3 times as active as sulphanilamide, whereas the sulphathiazoles seem 3–5 times as active as sulphapyridine. It is not the absolute value but the relative activity that has to be taken into account. Still the insensitivity of Bact. coli for sulphanilamide is a peculiar feature.

TABLE II

RELATIVE ACTIVITIES FOR VARIOUS SULPHANILAMIDE DERIVATIVES (SULPHANILAMIDE = 1)

I Sulphanilamide
II 2-Sulphanilylamino-4-methylpyrimidine
III Sulphapyridine
IV 5-Sulphanilylamino-4-methylpyrimidine
V Sulphathiazole
VI Sulphamethylthiazole

Bacteria studied	I	II	III	IV	V	VI	m.a.c. in milli-equivalents for sulphanilamide
1. Bact. coli; synthetic medium	1	17	30	40	75	85	3
2. Bact. coli; synthetic medium[1]	1	20	30	60	150	100	
3. Bact. coli; blood broth	1	16	12	40	75	40	1.2
4. Streptococci; blood broth	1	3	3	2	4	6	0.17
5. Streptococci; blood+NaCl	1	1.5	1.6	1	4.5	5	0.1
6. Staphylococci; semi-synthetic medium	1	2.3	4.5	2.3	15	16	0.17
7. Staphylococci; same medium, extinctiometric technique	1	3	6	6	70	30	(0.023)[2]
8. Pneumococci; blood broth	1	—	2.2	—	—	—	0.43

[1] As in later experiments the definition of the m.a.c. was slightly varied, the factors for Bact. coli had to be recalculated (from the same experiments as in Table 1) to permit comparison with other bacteria.

[2] The m.a.c. with turbidity as a measure of growth is absolutely incomparable with those from growth experiments as was discussed on p. 12, the relative values for the derivatives, however, even hold true in this case.

β. In vivo.

Considering the activity of sulphonamide derivatives there are two problems to discuss:

1°. What is the explanation of the different activity of various compounds against a given strain of bacteria?

2°. *Is there a specific action of some derivative for a special* kind of bacteria, e.g., sulphapyridine/pneumococci, or sulphathiazole/staphylococci?

Ad 1. The different activity of sulphanilamide compounds in vivo can be explained, at least partially, by the differences in activity already present in vitro. On the other hand the twofold increase in activity towards pneumococci as shown e.g., by sulphapyridine in comparison with sulphanilamide (Table II) can hardly explain the remarkable effectiveness of the former drug in pneumococcal infections. Still other factors seem to be at work here.

The chemotherapeutical effect of each drug will be dependent on the blood concentration which can be obtained, and the latter will be dependent on many factors. An upper limit will be defined by the toxicity of the drug, below this the blood concentration will be determined by supply (velocity of resorption from the intestine) and conveyance (velocity of excretion, acetylation, resorption into the tissues, etc.). Considering all these phenomena, which might moreover vary in each individual, it seems very well possible that a given drug in vivo is more potent than sulphanilamide, whereas in vitro no significant difference in activity can be established. Besides, the difference in sensibility of each special bacterial strain will exert its influence on the final results (compare page 51). Probably many a clinical observation on the exceptional value of a given drug, perhaps even the specificity claimed for some compounds, can be explained in this way.

So the observations on man and animals seem to be compatible with the general view that also in vivo the mechanism of action of the derivatives and of sulphanilamide is essentially the same.

On the other hand, the possibility always remains that with the introduction of a substituent (a pyridine nucleus or whatever else) into the sulphanilamide molecule, apart from enhancing the sulphanilamide action, another activity is given to the molecule. For sulphapyridine there is a case in point.

Julius and Salomon (1941), working on the latter supposition, were able to show that in pneumococcal infections in mice, which could be cured with sulphapyridine, but not by sulphanilamide, the action of sulphanilamide is enhanced by small doses of pyridine (orally or subcutaneously), which was inactive when given alone. Some pyridine derivatives like α-methylpyridine and benzenesulphonamidopyridine showed this effect in a more marked manner, while in the combination of sulphanilamide with toluenesulphonamidopyridine the effect of sulphapyridine was nearly equalled, 10 out of 10 mice surviving. The supplementing substance by itself was totally devoid of antibacterial capacity.

So it must be concluded that a supplementary activity is present in the action of sulphapyridine on pneumococci, as a synthesis of the drug from its constituents in the host is improbable; this can also be excluded from the supplementing activity of a substance like α-methylpyridine.

The mechanism of this other activity is not yet known, but it probably has to do with the defence of the host as no supplementing action of the named pyridine derivatives could be established in vitro.

It is interesting to add that, apart from the said compounds, not only quinine but also pyramidon and especially acetanilide showed the supplementing effect in vivo.

So in vivo the sulphanilamide derivatives are generally acting through the same (sulphanilamide-) mechanism. Their different activities are partly explained by differences in adsorption in the bacteria, partly by the phenomena which define the blood concentration that can be obtained. In some cases a second activity can be present in the molecule.

Ad 2. The second question need not be treated extensively.

From Table II it is evident that, at least in vitro, there exists no specific activity of e.g., sulphapyridine against pneumococci, or of sulphathiazole against staphylococci, as, apart from minor variations, the order of sequence in all cases is: sulphanilamide < sulphapyridine and sulphapyrimidines < sulphathiazoles.

A specific action of a given drug against a special kind of bacteria could thus exist only if a second activity were present in the molecule. It seems, however, very improbable that this action is specific. Acting on the mechanism of defence of the host it is much more likely to be of unspecific character. So the question is as yet undecided, although we are inclined to suppose that most of the clinical observations in this respect can be explained by the phenomena of resorption, excretion, toxicity and the like, and thus in general can be reduced to mere quantitative relations.

Summary.

1. The mechanism of the action in vitro of the derivatives of sulphanilamide appears to be the same as that of the mother substance.

2. In vitro the only difference between the various compounds is a difference in active concentration.

3. The different activity in vitro of various sulphanilamide derivatives is due to differences in degree of adsorption to the bacteria.

4. The mechanism of the action of various sulphanilamide derivatives in vivo in general, also seems to be the same as that of sulphanilamide.

5. The different activity of the derivatives in vivo can be explained: by differences in vitro (varying degree of adsorption), by differences in resorption, excretion, toxicity etc. (pharmacological properties), and
by a supplementary (unspecific) activity introduced by the heterocyclic nucleus (as demonstrated for sulphapyridine in the case of pneumococci).

6. A specific activity of a given drug against a special kind of bacteria (sulphapyridine/pneumococci; sulphathiazole/staphylococci), does not exist in vitro. Though clinical observations seem to suggest such a relation in vivo, in many cases this can be reduced to quantitative differences.

g. The problem of chemotherapeutic resistance.

The problem of chemotherapeutic resistance is a problem of its own, the solution of which may contribute considerably to the insight into the mechanism of the action of the drugs. So a short discussion on the resistance of the sulphonamides, though little supported by experimental data, may find its place here.

A number of infectious diseases as well as a number of individual cases of diseases, which are capable of being cured, are resistant to sulphonamide medication. These cases are by no means identical. For the sake of avoiding confusion we will be obliged to analyze and to subdivide the matter.

a. *Primary resistance.*

1. The absolute type.

The greater part of the virus infections (but not all of them: lymphogranuloma inguinale) and nearly all protozoal infections (except perhaps malaria, v. d. Wielen, 1937) are refractory to sulphonamide therapy. Analogous observations are as old as chemotherapy itself: quinine is active towards malaria, but not towards trypanosomiasis, salvarsan will cure syphilis and yaws, it fails to cure Weil's disease. Within this scope "resistance" is identical with the wellknown so-called "specificity" of chemotherapeutics.

Sulphanilamide is considered to act somewhere on bacterial metabolism. In one or more respects this will be different from the metabolic processes in relation to virus reproduction or from protozoal metabolism. The "absolute" type may thus be due to a lack of the point of attack for the sulphonamides in the insensible infectants (protozoa, viruses, Rickettsiae, etc.). As Ehrlich introduced the conception of "atreptic" immunity (insensibility) it was an allusion to the same supposition. If we consider the relatively great insensitiveness of the animal body-cells to the sulphonamides (e.g., in tissue cultures, Wolff and Julius, 1939) as compared with the bacterial cells we may consider this conception as sufficiently well founded for preliminary acceptance. But at the same time we must admit that adequate material of concrete data is not at our disposal.

Still we must put the question whether the primary resistance of bacteria may be of the absolute type. Might it be possible that there are bacteria devoid of the point of attack of the sulphonamides, viz., that the metabolic mechanism common to the greater part of the bacterial and even metaphytal world is lacking? Or might this mechanism be less essential to them or perhaps be interchangeable with another path of metabolic possibilities, able to replace a blocked line? Here again we must state that we are in want of data.

2. The relative type.

Considering the above conception of absolute primary resistance, the resistance of infectants will be relative if it can be shown that the point of attack is present.

In many refractory infections such as typhoid fever, paratyphoid, anthrax, tuberculosis, diphtheria, a.o., sulphanilamide medication, though not of clinical value, is not totally devoid of beneficial effect. Other infections, though refractory to sulphanilamide, are readily cured by sulphapyridine and other heterocyclic derivatives, which have the same point of attack as sulphanilamide (page 44). For a great number of clinically important strains of bacteria it is known that they are "sulphanilamide sensitive" when tested in vitro. These three features show that the point of attack is present in vitro, and in vivo in those cases their resistance must be of the relative type.

Is it possible, then, by comparing the minimal active concentrations for various infectants in vitro (under similar conditions) to analyze resistance in vivo? Though extensive material on this subject is still lacking, the experiments available show that already in vitro there exist differences in resistance which are not of the secondary type. Evidently resistance in vitro is a mere question of concentration. We might assume that the point of attack has not always the same vital significance for every strain of bacteria, another path of metabolism being at least partly interchangeable with the blocked line, though other explanations could be constructed.

In spite of the fact that many authors have found a rather close relation between the resistance of infectants in vivo and the relative resistance of the infecting strain in vitro, it is evident that the relatively insignificant differences found in vitro are generally unable to explain the wide scale of resistance in vivo, ranging from the very sensitive hemolytic streptococci to the resistant infections already mentioned.

The limited concentrations, realizable in vivo might offer an explanation. The fact that many of these infections are incurable by sulphonamides, but that death is often unmistakably retarded by the medications, points in this direction. The beneficial effect of sulphapyridine and other heterocyclic derivatives, where sulphanilamide fails, show that also in vivo resistance is a question of concentration.

Furthermore we have to realize that, for equal concentrations of sulphanilamide, even in vitro, small alterations in the medium (comp. p. 25) seriously affect sulphanilamide action. The influence of temperature, activators and inhibitors evidently will not be the same for different infectants to the same extent. Also the defence of the host will constitute a growth inhibitor which largely varies with one infectant to the other, quite apart still from the unequal distribution of different bacteria (and of the drug) over different organs.

Still other factors seem to play a part if we consider the resistance of e.g., pneumococci. The primary relative resistance of the pneumococcus as compared with the streptococcus after our in vitro studies is so small that it is quite impossible to explain the fact that pneumococcal infections in mice will not be cured by a dose of sulphanilamide more than 50 times the dose needed for curing streptococcal infection. We showed (page 53), that for the action of sulphapyridine on pneumococci an influence of the pyridine component on the defence of the infected host had to be accepted. So we are justified in asking, if this primary and relative resistance of some kinds of bacteria (pneumococci) is a special property of the cocci in vivo (capsule formation, permeation through the capsules), or if it may be combined with some special relation to the defence of the host.

Another interesting instance of the complexity of the problem of relative resistance is the cystitis caused by the colon bacillus, which is rather satisfactorily influenced by sulphonamide therapy. If the colon bacillus is tested in vivo, however, (e.g., Fig. 14), it appears to be several times less sensitive to sulphanilamide than the streptococcus (Table II, page 51). However, it has to be kept in mind that urine is a very special medium, which might be poor in inhibitors and even contain activators. This is not an empty supposition as indeed the minimal active concentration of sulphanilamide on bacterium coli in vitro with urine as a culture medium is 5–10 times smaller (about 0.1 $^0/_{00}$) than in the synthetic medium used in Fig. 14.

Generally the relative sensitiveness in vitro is the criterion for relative resistance in vivo, on the other hand there are so many complicating factors in vivo that finally each case will have to be studied separately.

β. Secondary resistance.

As chemotherapy was born, almost simultaneously research was startled by what Ehrlich described as "Arzneifestigkeit". Primarily sensitive bacteria may also, after some inexact treatment, become resistant to the drugs of the sulphonamide group. We should like to designate this acquired form as a secondary resistance. It took of course a certain time of sulphanilamide medication before the phenomenon was described, but we only have to understand what is happening with the gonococcus to be greatly alarmed in this respect. For how long will it be limited to the gonococcus?

But apart from its clinical urgency, it is of scientific value, as it is very well conceivable that the phenomenon of (secondary) resistance offers some clue to the mechanism of sulphonamide action in general.

The first essential fact which was demonstrated is, that the sulphonamide resistance can be induced by any member of the group and holds for the whole group. In the track of the work of MULDER (1940), JULIUS and WINKLER (unpublished) succeeded in producing a strain of pneumococcus (type I) resistant to sulphapyridine by insufficient treatment of mice infected with the strain. After every successive treatment the resistance increases, till after sixteen re-inoculations and undertreatments a strain was obtained that was refractory even to the highest doses of sulphapyridine realizable. The same could be achieved by undertreatment with sulphanilamide, the result being a strain totally refractory to sulphapyridine and other sulphonamides (tested: sulphathiazole), even in high concentration. Sixteen passages through the mouse were needed to obtain this result. The same, though rather less easily, could be realized in streptococci. A strain resulted that induced infections incurable by sulphapyridine.

In vitro experiments with streptococci, made resistant in vivo, showed that in vitro also the resistance had increased. In quantitative experiments with normal and resistant streptococci the curves for a series of concentrations were similar for both strains, the only difference being the concentrations needed. And though an extensive experimental analysis is still lacking, the general inference is that secondary resistance in vivo and in vitro run parallel, though several exaggerating factors may influence the resistance in vivo.

The p.a.b.a. hypothesis of sulphonamide-action soon gave rise to the supposition that secondary (adaptative) resistance might be caused by the increased production of p.a.b.a. by the resistant strain, which, to a certain degree, is supported by the fact that Mc.Leod (1940) demonstrated that the antisulphonamide activity of a culture of a resistant strain of pneumococcus (Type I) was stronger than that of a normal strain, and Green and Bielschowsky (1942) could prove the same for Bac. paramelitensis. Landy, Larkum, Oswald and Streightoff (1943) produced more direct evidence for the same view by showing that sulphonamide resistant strains contained more p.a.b.a. than the corresponding normal strains.

If an excessive production of p.a.b.a. might account for the resistance in resistant strains, a smaller concentration of p.a.b.a. should be necessary to inhibit the sulphanilamide action, comparing equal concentrations of sulphanilamide acting on the normal and resistant strains. On the other hand an equal amount of p.a.b.a. should be necessary if one compared different amounts of sulphanilamide having an equal effect on both strains. Indeed the results of our experiments were in accordance with this reasoning as is shown in Fig. 18.

The log of count after 24 hours is plotted here against the log of the concentration of p.a.b.a. used. The curve for the resistant strain starts at a higher level, the number of bacteria for the resistant strain with sulphanilamide only, does not fall below 10.000 (per standard droplet) or log of count 4. As log of count 4 (after 24 hours) with the normal strain is obtained only in the presence of 0.0002 milli equivalents of p.a.b.a., one might consider the resistant strain as a normal strain producing in the cocci an amount of p.a.b.a. equalling the effect of an external p.a.b.a. concentration of 0.0002 milli equivalents. Acting on this supposition, the addition of 0.0002 milli equivalents to the concentrations of p.a.b.a. plotted for the resistant strain led to the points +, which practically coincide with the curve for the normal strain.

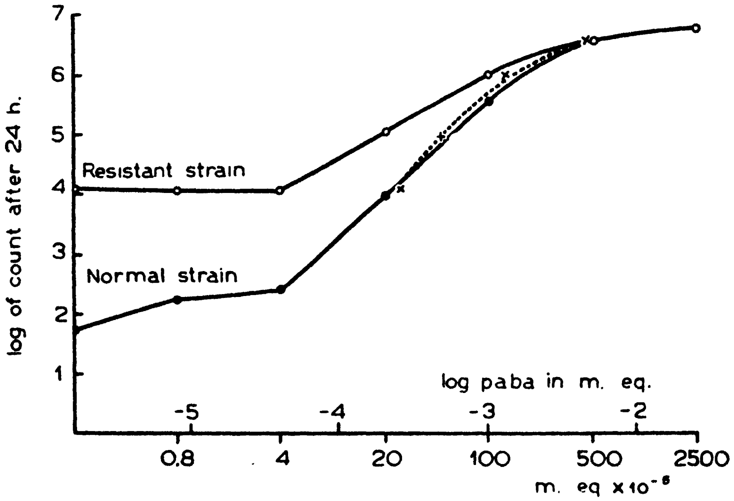


Fig. 18. Influence of p.a.b.a. on the growth of normal and resistant strains of streptococci in cultures with 0.015 % sulphanilamide.

Ivanovics (1941, 1944) proved that under the conditions of his test for a certain strain of staphylococcus, one mole of p.a.b.a. was able to annihilate the activity of 14 moles of sulphathiazole, while for the resistant strain this proportion was 1 : 220.

So in both cases the experimental evidence is in accordance with the view that the resistance to a sulphonamide (of the studied strain) is due to the production of a certain amount of antagonist of the p.a.b.a. type.

Concluding we may say that primary absolute resistance is probably due to the lack of a point of attack, primary relative resistance is due to a great many causes of which the greater part is not as yet understood, whilst secondary resistance in several cases is due most probably to an excessive production of p.a.b.a.

LITERATURE

Cited on page

AUHAGEN, E., *p-Aminobenzoylglutaminsäure, ein gegen Sulfonamide wirksameres Derivat des Vitamins* H'. H. S. Zeitschr. f. physiol. Chem, 277 (1943) 197 . . . . 31, 32

BARKER, H. A., *The oxidative metabolism of the colourless alga, Prototheca Zopfii.* J. Cell. Comp. Pyshiol., 8 (1936) 231 19

BLISS, E. A. AND LONG, P. H., *Observations on the mode of action of sulfanilamide. The antibacteriostatic action of methionine.* Bull. John Hopkins Hosp., 69 (1941) 14 31

CLIFTON, C. E., *On the possibility of preventing assimilation in respiring cells.* Enzymologia, 4 (1937) 246 19

COLEBROOK, L., BUTTLE, G. A. H. AND O'MEARA, R. A. Q., *The mode of action of p-aminobenzene sulphonamide and prontosil in haemolytic streptococcal infections.* Lancet (1936) II 1323 10

DOMAGK, G., *Ein Beitrag zur Chemotherapie der bakteriellen Infektionen.* Dtsch. Med. Wochenschr., 61 (1935) 250 10

DOMAGK, G., *Chemotherapie der bakteriellen Infektionen.* Ang. Chemie, 48 (1935) 657 10

FELDT, A., *Die Chemotherapie der Recurrensinfektion mit Gold- und Sulfonamidverbindungen.* Klin. Wochenschr., 20 (1941) 945 . . . 24

FILDES, P., *The mechanism of the antibacterial action of mercury.* Brit. J. exp. Path., 21 (1940) 67 23

FINKELSTEIN, R. AND BIRKELAND, J. M., *The mode of action of sulfanilamide and prontosil.* Science, 87 (1938) 441 10

FINKLESTONE-SEYLISS, H., PAINE, C. G. AND PATRICK, L. B., *The bacteriostatic action of p-aminobenzenesulphonamide upon haemolytic streptococci.* Lancet (1937) II 792 15

FREI, W., *Oxydationsvorgänge bei Bakterien und ihre Bedeutung für Pathogenese und Chemotherapie.* Schweiz. med. Wochenschr., 74 (1942) 69 18

Cited on page

Giesberger, G., *Beiträge zur Kenntnis der Gattung Spirillum Ehbg.* Thesis Delft (1936) 19

Goor, H. van, *The inhibition of carbonic anhydrase by sulphanilamide.* Onderzoekingen Physiol. Lab. Rijksuniversiteit Utrecht (1942) (8) II, 142. To be published in Enzymologia XI 24, 25

Green, H. N. and Bielschowsky, F., *Brit. J. exp. Path.*, 23 (1942) 1. Cited from Ivánovics (1944) 59

Harris, J. S. and Kohn, H. I., *J. pharm. exp. Ther.*, 73 (1941) 383. Cited from Ivánovics (1944) 31, 41

Hirsch, J., *Studien über die mikrobiologischen Grundlagen der Sulfanilamidtherapie.* Compt. rend. annu. Arch. Soc. turque Sci. phys. natur., 10 (1941/1942) 115. C (1942) II 1933 . . . . . . 12, 18

Hirsch, J., *Die „parasitotropen" Eigenschaften der Sulfanilamide.* Schweiz. Med. Wochenschr., 73 (1943) 1470 12

Ivánovics, G, *Ueber den Wirkungsmechanismus des p.Aminobenzolsulfonamids und seiner Derivate.* Z. f. Immunitätsforsch., 101 (1941) 58 60

Ivánovics, G., *Experimentelle Chemotherapie der bakteriellen Infektionen.* Acta Medica Szeged (1944) Tom. XII, Fasc. 1. . . . . 32, 50, 60

Janssen, L. W., *Biosynthesis and the outlines of protein structures.* Protoplasma, 33 (1939) 410. 22

Jensen, K. A., Schmith, K. and Brandt, P., *Über die bakteriostatische Wirkung von p.(Aminomethyl-)benzolsulfonamid* Klin. Wochenschr., 21 (1942) 1042 44

Julius, H. W. *Een methode voor het tellen van levende bacteriën, vervanging voor de plaatmethode.* Ant. v. Leeuwenhoek, 5 (1938) 28 . . 12

Julius, H. W. and Salomon, A., *On the action of sulfanilamide III. The difference between sulfanilamide and sulfapyridine.* Ant. v. Leeuwenhoek, 7 (1941) 77 53

Julius, H. W. and Winkler, K. C., *On the action of Sulfanilamide II. The action of sulfanilamide on catalase.* Ant. v. Leeuwenhoek, 7 (1941) 25 18

Julius, H. W. and Winkler, K. C., *On the action of sulfanilamide IV. Is the sulfanilamide molecule altered before action?* Ant. v. Leeuwenhoek, 7 (1941) 153 (a) 17

Julius, H. W. and Winkler, K. C., *On the action of sulfanilamide VIII. The mechanism of its action.* Ant. v. Leeuwenhoek, 8 (1942) 139. 23, 24, 31

Julius, H. W. and Winkler, K. C., To be published in Ant. v. Leeuwenhoek, 1945 45

Kohn, H. I. and Harris, J. S., *The mechanism of the action of the sulphonamides I. Action on Escherichia coli.* J. Pharmacol. exp. Ther. 73 (1941) 343; C (1943) II 936 18

Kuhn, R., Wieland, Th. and Möller, E. F., *Synthese des (α.γ-dioxy-β.β-dimethyl-butyryl)-taurins, eines spezifischen Hemmstoffes für Milchsäurebakterien.* Ber., 74 (1941) 1605 23

Cited on page

Kuhn, R. and Schwarz, K., *Isolierung des Wuchsstoffes H' aus Hefe.* Ber., 74 (1941) 1617 23

Kuhn, R., Möller, E. F., Wendt, G. and Beinert, H., *4,4'-Diaminobenzophenon und andere schwefelfreie Verbindungen mit Sulfonamidwirkung.* Ber., 75 (1942) 711 23

Landy, M., Larkum, N. W., Oswald, E. J. and Streightoff, F., *Increased synthesis of p-aminobenzoic acid associated with the development of sulfonamide resistance in Staphylococcus aureus.* Science, 97 (1943) 265 59

Levaditi, C. and Vaisman, A., *Action curative du chlorhydrate de 4'-sulfamido-2,4-diaminoazobenzène et de quelques dérivés similaires, dans la streptococcie expérimentale.* C. R. Soc. Biol., 119 (1935) 946; comp. Presse Méd., 43 (1935) 2097 10

Levaditi, C. and Vaisman, A., *Mécanisme de l'action curative et préventive du chlorhydrate de 4'-sulfamido-2,4-diaminoazobenzène et d'autres dérivés similaires dans l'infection streptococcique expérimentale.* C. R. Soc. Biol., 120 (1935) 1077 10

Levaditi, C., *La chimiothérapie des infections microbiennes. Son mécanisme d'action.* Schweiz. Ztschr. allg. Path. Bakt. I (1938) 365 10

Locke, A., Main, E. R. and Mellon, R. R., *On specific factors in resistance. III. Capacity for retarding bacterial proliferation.* J. Immunol., 36 (1939) 183. 17

Mann, T. and Keilin, D., *Sulphanilamide as a specific inhibitor of carbonic anhydrase.* Nature, 146 (1940) 164 24

Martin, G. J. and Fisher, C. V., *Antisulphonamide activity of adenin, 6-aminopurin.* J. Biol. Chem., 144 (1942) 289; C. (1943) I, 1294 31

McLeod, C. M., *Inhibition of bacteriostatic actior of sulfonamide drugs by substances of animal and bacterial origin.* J. exp. Med., 72 (1940) 217 59

Mietsch, F. and Klarer, J., D. R. P. 607537 (1935) 10

Möller, E. F. and Schwarz, K., *Der Wuchsstoff H', ein Antagonist der Sulfanilamide bei Streptobacterium plantarum* (Orla-Jensen); *Wachstum von Str. plantarum in Nährlösungen aus chemisch genau definierten Verbindungen.* Ber., 74 (1941) 1612. 22

Mudrow, L. and Bock, M., *Der Antagonismus von Sulfonamiden und Para-aminobenzoësäure bei experimentellen Spirochäten-, Spirillen- und Virusinfektionen.* Z. f. Immunitätsforsch., 104 (1943) 463 . . . 24

Mulder, J., *Het ontstaan van sulfanilamidopyridine-vaste pneumococcus-stammen in vivo.* Ned. Tijdschr. v. Geneesk., 84 (1940) 923 . . . 58

Punt, A., *De invloed van sulfanilamide op de weefseloxydatie in vitro.* Ned. Tijdschr. v. Geneesk., 87 (1943) 1116. 18

Quastel, J. H. and Woolf, B., *The equilibrium between l-aspartic acid, fumaric acid and ammonia in the presence of resting bacteria.* Bioch. J., 20 (1926) 545 20

Cited on page

Rose, H. M. and Fox, Ch. L., *A quantitative analysis of sulphonamide bacteriostasis.* Science, 95 (1942) 412. 50

Rubbo, S. D. and Gillespie, J. M., *Para-aminobenzoic acid as bacterial growth factor.* Nature, 146 (1940) 838 23

Snell, E. E., *Reversal of a specific growth inhibition by pantothenic acid.* J. Biol. Chem., 139 (1941) 975 23

Snell, E. E., *Growth inhibition by N-(α,γ-dihydroxy-β,β-dimethylbutyryl) taurine and its reversal by panthothenic acid.* J. Biol. Chem., 141 (1941) 121 23

Söhring, K., *Stoffwechselwirkungen der Sulfonamidverbindungen an Mikro-Organismen. Über den Einflusz der Sulfonamide auf den Sauerstoffverbrauch von Bact. coli und Bäckerhefe in synthetischen Medien, sowie über Beziehungen zwischen Stoffwechsel- und Wachstumshemmung.* Ztschr. f. ges. exp. Med., 111 (1942) 585 18

Tréfouël, J., Tréfouël, Mme, J., Nitti, F. and Bovet, D., *Activité du p-aminophénylsulfamide sur les infections streptococciques expérimentales de la souris et du lapin.* C. R. Soc. Biol., 120 (1935) 756. 10

White, H. J., *The relationship between temperature and the streptococcidal activity of sulfanilamide and sulfapyridine in vitro.* J. Bacteriol. 38 (1939) 549 33

Wielen, U. van der, *Prontosil en malaria quartana.* Ned. Tijdschr. v. Geneesk., 81 (1937) 2905 55

Winkler, K. C., *On the action of sulfanilamide VI. The action on bacterial respiration.* Ant. v. Leeuwenhoek, 8 (1942) 10 18

Winkler, K. C., *On the action of sulfanilamide IX. The action on the synthesis of amino-acids by bacteria.* Ant. v. Leeuwenhoek, 9 (1943) 115 . . . . 19, 20

Winkler, K. C. and Julius, H. W., *On the action of sulfanilamide V. The action on anaerobic growth.* Ant. v. Leeuwenhoek, 7 (1941) 161. 18

Winkler, K. C. and Julius, H. W., *On the action of sulfanilamide. VII. A sulfanilamide activating substance in horse blood.* Ant. v. Leeuwenhoek, 8 (1942) 86 34

Winkler, K. C. and Julius, H. W., *Über den Wirkungsmechanismus der Sulfonamide. XI. Über den Wirkungsmechanismus des p-Aminomethyl-benzolsulfonamids (Marfanil).* Schweiz. Ztschr. f. Pathol. und Bakteriol. VI (1944) 455 44

Wolff, L. K. and Julius, H. W., *Action du sulfanilamide in vitro et in vivo.* Ann. Inst. Pasteur, 62 (1939) 616 . . . . 11, 15, 34, 56

Woods, D. D., *The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide.* Brit. J. exp. Path., 21 (1940) 74 . 22, 23, 36

§ 2. PHYSICO-CHEMICAL INVESTIGATIONS

As already mentioned in bacteriological investigations, important starting points for the question of the mechanism of action of the sulphonamides were discovered first by investigators, who occupied themselves in this field too with chemotherapeutic interference phenomena.

By the researches of FILDES (1940), WOODS (1940) and others the hypothesis came to the fore that p-aminobenzoic acid, acting as an antagonist with respect to sulphonamides, would be an essential metabolite and that then the action of the sulphonamides had to be ascribed to a displacement of the p-aminobenzoic acid from the place (e.g., apoferment) where it exercises its activity.

The results of the investigations of RUBBO and GILLESPIE (1940), BLANCHARD (1941) and of KUHN and SCHWARZ (1941) formed such a support to this attractive hypothesis, that it was accepted almost without criticism (comp. VELDSTRA, 1942). This version, however, was questioned by JULIUS and WINKLER (1942), MARKEES (1943) and also by LÄUGER et al. (1944), moreover, no detailed representations about character and place of the action of p.a.b.a. and the sulphonamides have been developed till now.

While JULIUS and WINKLER, in pursuing this object, started particularly from the results of their bacteriological experiments (see page 10), HAVINGA and VELDSTRA, in a number of physico-chemical researches on p.a.b.a., sulphonamides and related compounds (HAVINGA and VELDSTRA, A, B; VELDSTRA and HAVINGA, A, B) have looked for starting-points to gain a deeper insight in this question, which is alike of so much importance to the apprehension of the action of ergons and antivitamins in general.

a. Electrometric titrations.

Electrometric titration curves were recorded of o-, m- and p-aminobenzoic acid and two related compounds, further of sulphanilic acid, sulphanilamide and a number of its heterocyclic derivatives. The purpose was to obtain data about the basic and acid groups occurring in their molecules, and of comparing p.a.b.a. in this respect with the sulphonamides and the sulphonamides among each other, especially to determine possible differences between sulphanilamide and the heterocyclic derivatives.

The character of the amino-groups was established by the previous addition of one equivalent of hydrochloric acid. *In a number of cases the titrations were carried out in dilute alcohol, on account of the solubility of the compounds. The results of the measurings are represented in Figures 19 and 20.*

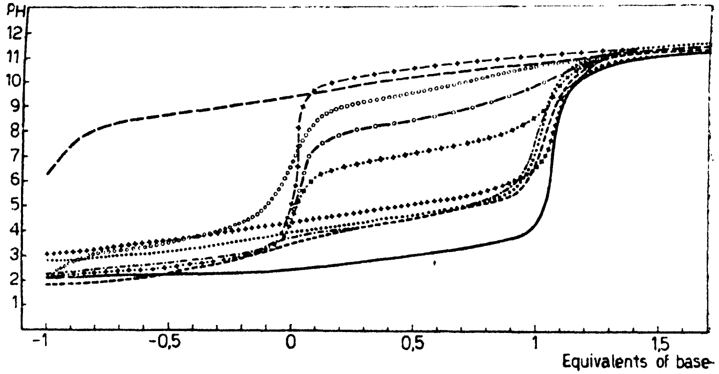
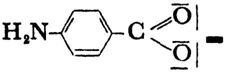
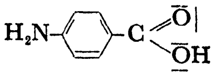


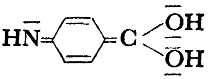
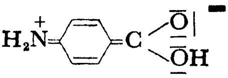
Fig. 19. Electrometric titrations (in aqueous solution). Before the titration an equivalent amount of hydrochloric acid was added.

- - - - - - - - -	o-aminobenzoic acid
···················	m-aminobenzoic acid
—·—·—·—·—	p-aminobenzoic acid
+++++++++++	p-aminophenylacetic acid
ooooooooooo	p-(aminomethyl-)benzoic acid
————————	sulphanilic acid
+—+—+—+—	sulphanilamide
-o—o—•—•—	sulphapyridine
+·+·+·+·+·+	sulphathiazole
— — — — — —	p-(aminomethyl-)benzene sulphonamide (Marfanil)

Regarding the structure of p-aminobenzoic acid, it may be concluded that, under physiological circumstances, it is chiefly present in the form of negative ions with respect to the carboxyl group; besides this, a small part is not ionized. So, contrary to the opinion often expressed, p.a.b.a., in aqueous solution, for-the greater part is not present as dipole-ions (or "Zwitterions"). As the basic character of the amino-group appears to be very weak (compare also the ultraviolet absorption spectra), these structures can be reproduced as follows:


,


In theoretical speculations other structures have to be taken into account, e.g., for the undissociated acid a protomer:


and an electromer:


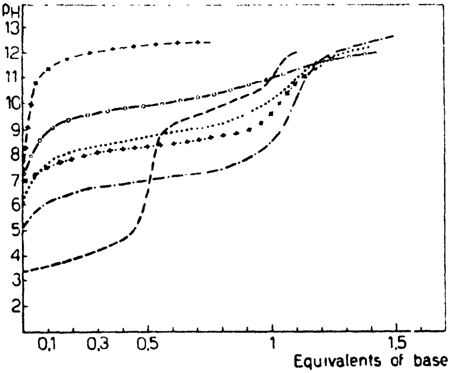


Fig. 20. Electrometric titrations (in 60 proc. alcohol).

+·+·+·+·+·+·+ sulpha-methylpyrimidine
- - - - - - - - - sulphatetrazole
·—·—·—·— 1-aminonaphthalene-4-carbonic acid
·—+—+—+— naphthionamide
o—o—o—o—o— 2-naphthionylamino-pyridine
.... 2-naphthionylamino-4-methylpyrimidine

(compare also the data on ultraviolet absorption spectra).

As to their acid function o- and m-aminobenzoic acid are quite comparable with p.a.b.a., the amino-group however is somewhat less basic in the ortho isomer and a little stronger in the meta isomer. On account of the physiological inactivity of the o- and m-compounds, the character of the amino-group is, besides other — perhaps sterical — factors, obviously more determinant for the physiological function than that of the carboxyl group. A great number of other facts (also applying to the sulphonamides) point to this, and will be discussed further.

While p-aminophenylacetic acid is a somewhat weaker acid than p.a.b.a., the basic character of its amino-group is a little stronger. The general aspect of the curve is not essentially different from that of the three aminobenzoic acids. p-(Amino-methyl-)benzoic acid, however, we should suppose to be present as dipole ions ("Zwitterions") in neutral solution. This hypothesis explains the totally different titration curve. Thus separation of the amino-group from the benzene nucleus by a $-CH_2-$ group involves more essential changes than if this happens with the carboxyl group (compare the curves). This is of importance to the question of the properties of marfanil, to be discussed below.

If sulphanilamide is compared with p.a.b.a., the character of their amino-groups proves to be nearly identical, sulphanilamide however is a much weaker acid. Of the heterocyclic sulphanilamide derivatives the aminogroup in sulphapyridine, sulphathiazole and sulpha-methylpyrimidine is not noticeably influenced by the substitution, the acid character of the $-SO_2NH-$ grouping has increased however, though it is considerably lower than that of p.a.b.a. This involves that the heterocyclic derivatives will have a somewhat stronger polar character than sulphanilamide, as has already appeared from measurings of the dipole moment by JENSEN and FRIEDIGER (1942).

The fact that the difference between p.a.b.a. and the sulphonamides and between the sulphonamides mutually, lies in the acid function, while their "basic" behaviour is remarkably alike, again suggests — as in the comparison of the aminobenzoic acids — that particular importance is due to the amino-group, probably for the chemotherapeutic properties of the sulphonamides as well as for their interaction with p.a.b.a. activity. On account of the determination of the relative acidity and basicity of sulphanilamide and p.a.b.a., ALBERT and GOLDACRE (1942) expressed the same supposition. The well-known experience that substitutions in the amino-group of the sulphonamides decrease or undo the activity (comp. e.g., Northey, 1940; Jensen and Schmith, 1942, 1944; Rune Frisk, 1943) points in the same direction.

Strong acid character (sulphanilic acid, sulphatetrazole) results in very low chemotherapeutic activity. The most active sulphonamides thus occupy an average position as to their acid function.

Bell and Roblin (1942) consider the acidity of the sulphonamides highly determinant for their activity. On account of this research and the relation, supposed by Kimmig and Weselman (1941), and Davis and Wood (1942) respectively, to exist between the action of the sulphonamides and their faculty of complex formation with proteins, Klotz (1943) formulated this action in dependence on dissociation-constant and tendency towards complex formation. He arrived at the conclusion that for strong activity an average value of the dissociation-constant is required, as the rate of complex formation is maximal then. This complex formation namely is supposed to take place between a sulphonamide anion as a base (rôle of the NH_2-group!) and an acid group of an enzyme protein, so that with weakly acid sulphonamides the low anion content, and with strongly acid ones the weakly basic character of the anion will bring on complex formation to a lower extent (at p_H 7) (comp. Ivánovics, 1944, p. 104–105).

Without drawing these ideas into the discussion, we may conclude that, on the ground of our measurings, we arrive at the same conclusion as Klotz, regarding the acid character of the strongest active sulphonamides.

As appears from the titration curves the sulphonamide groups of marfanil and sulphanilamide are pretty well comparable, the aliphatic amino-group of marfanil however is much stronger basic than the amino group of sulphanilamide and its derivatives (compare "ultraviolet spectra"). Therefore the substance possesses a character, quite different from the sulphonamides, which also finds expression by the facts, that marfanil isn't counteracted by p.a.b.a. and contrary to the sulphonamides doesn't show a lag-time of action, but is acting directly (compare Jensen, Schmith and Brand, 1942 and Winkler and Julius, 1944).

The results of these electrometric titrations can be summarized as follows:

1. Ortho-, meta- and para-aminobenzoic acid are nearly equivalent as acids, the (minor) differences are due to the basicity of the amino group, which is the highest in the meta- and the lowest in the ortho-compound.
2. While p-aminophenylacetic acid does not essentially differ from the aminobenzoic acids, p-(aminomethyl-)benzoic acid behaves quite differently, forming dipole ions ("Zwitterions") to a much greater extent.
3. The behaviour of the amino-groups in p.a.b.a. and in the sulphonamides is quite alike. The acidity of the sulphonamides is considerably lower than that of p.a.b.a.
4. The heterocyclic derivatives of sulphanilamide are more strongly acid than sulphanilamide itself. The chemotherapeutically highest active derivatives occupy an intermediate position with respect to the acid character, as strongly acid compounds like sulphanilic acid and sulphatetrazole are but slightly active.
5. Marfanil behaves quite differently from sulphanilamide and its derivatives, owing to the much stronger basic character of its amino-group.

b. Ultraviolet absorption spectra.

The absorption curves of a number of the compounds investigated by HAVINGA and VELDSTRA (A) are represented in the Figures 21, 22 and 23. The spectra were recorded with the aid of a Hilger Barfit Medium Quartz Spectrograph and a Spekker photometer. The substances were dissolved in water and the thickness of the layer was 1 cm.

An ample discussion on the curves obtained is outside the scope of this book; moreover, in spite of the great progress that has been made of late in the matter of interpreting absorption spectra, it is still very risky to draw far-reaching conclusions in this way about the finer structure and the electronic configuration of relatively complicated compounds.

On contemplating the curves it is striking that p-aminobenzoic acid and the sulphonamides — besides showing a strong absorption at wave lengths shorter than 230 mμ — all show a marked absorption-maximum in the range of 250–280 mμ. Of all these compounds the extinction at this maximum is practically the same ($\varepsilon_{\text{mol}} = 1{,}5 \cdot 10^4$).

It seems justified to hold a certain resemblance in electronic configuration responsible for this, especially as the o- and m-aminobenzoic acids show a much lower extinction, moreover the maximum has shifted here to the longer wave lengths (compare also Dede and Rosenberg, 1934)).

Marfanil too, in which the NH_2-group is separated from the benzene nucleus by a $-CH_2$-group, has a much lower extinction. So the strong resemblance in chemical structure between p-aminobenzoic acid and the sulphonamides, which, from the very beginning, strongly interested investigators like Woods and Fildes, also stands out in the electronic configuration.

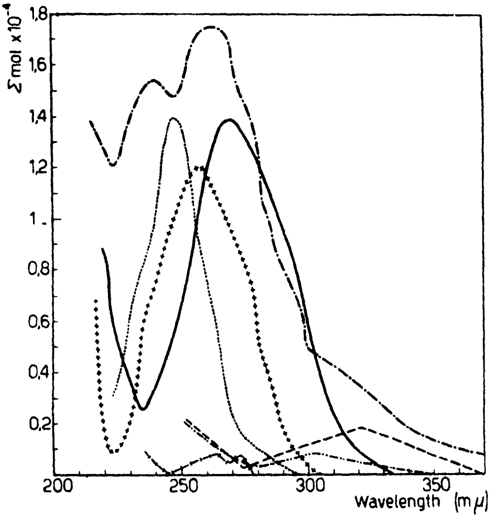


Fig. 21. Ultraviolet absorption-spectra.

- - - - - - -	o-aminobenzoic acid
- ·— · —··—·-	m-aminobenzoic acid
————————	p-aminobenzoic acid
··························	sulphanilic acid
+ + + + + + + + +	sulphanilamide
+—ı—ı—ı—ı—	p-aminomethyl-benzenesulphonamide (Marfanil)
—·—·—·—·—	sulpha-4-methylpyrimidine (Percoccide)

On closer examination of the spectra of the heterocyclic sulphanilamide derivatives, it is evident that introduction of a heterocyclic nucleus causes no essential change in the character of the absorption, though a distinct influence can be shown, which expresses itself by a broadening of the absorption band and the appearance of secondary maxima.

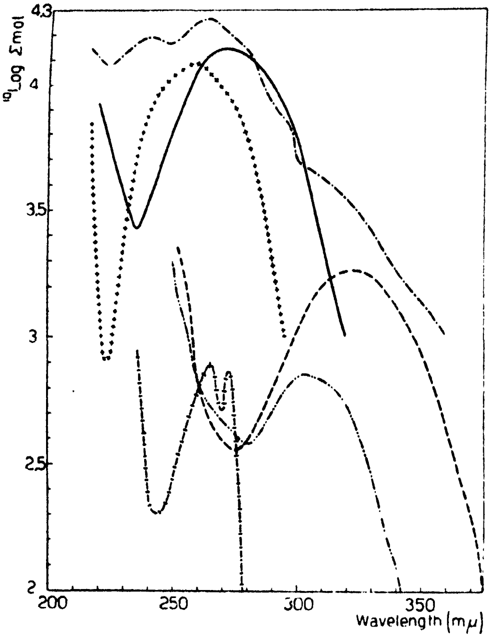


Fig. 22. Ultraviolet absorption-spectra.

– – – – – – –	o-aminobenzoic acid
—·—·—·—	m-aminobenzoic acid
————	p-aminobenzoic acid
++++++++++	sulphanilamide
–ı–ı–ı–ı–	p-aminomethyl-benzenesulphonamide (Marfanil)
—·—·—·—	sulpha-4-methylpyrimidine (Percoccide)

It is remarkable that from the five curves represented (Fig. 23), those of the chemotherapeutically strongly active substances show, besides the principal maximum, a by-maximum on the side of the shorter wave lengthts, while sulphatetrazole and sulphamethyl-pyridine do not display this phenomenon.

It is interesting to compare the curves of the (active) sulphapyridine with that of the (inactive) sulphamethyl-pyridine, in which pyridine-ring and sulphanilamide-grouping are not joined directly, but by a $-CH_2-$group. One is inclined to conclude that in sulphapyridine the influencing of the sulphanilamide spectrum by the pyridine nucleus (and the interaction of this ring with the sulphanilamide-part of the molecule, which is at least partially responsible too for the increased activity) is stronger than in the case of sulphamethyl-pyridine.

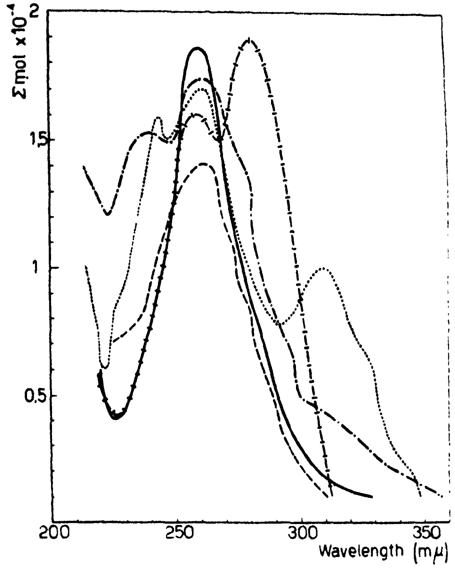
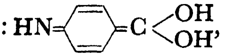


Fig. 23. Ultraviolet absorption-spectra.

.... sulphapyridine
–ı–ı–ı–ı–ı– sulphathiazole
—·—·—·— sulpha-4-methylpyrimidine
————— 2-(sulphamethyl)-pyridine
·– – – – – – – – 5-sulphatetrazole

The high extinction of p-aminobenzoic acid and the sulphonamides indicates that in water (p_H 6–7) the NH_2-group of these compounds is practically not dissociated; for, it was already known from experiments of former investigators (Riegel and Buchwald (1929); Dede and Rosenberg (1934); Pestemer and Flaschka (1938), that the change from $-C_6H_4-NH_2$ to $-C_6H_4-NH_3^+$ involves a sharp fall of the extinction. Very probably the low extinction of m-aminobenzoic acid and of marfanil will have to be ascribed to this too.

Thus, contrary to the opinion often expressed, p-aminobenzoic acid and the sulphonamides practically do not occur as a "zwitterion". For p-aminobenzoic acid this has been established earlier indeed on account of the dielectric studies of Hedestrand (1928); Devoto (1934) and Halbedel (1936). The high extinction of p-aminobenzoic acid and its esters lead Riegel and Buchwald (1929) to the supposition, that most of the molecules are in the hemiquinoid form, indicated by the formula: , which view is confirmed by the low absorptive power of p-dimethylaminobenzoic acid. An exact proof for the existence of this structure is however lacking. Moreover, with a sample of pure p-dimethylaminobenzoic acid, prepared from p-dimethylaminobenzaldehyde, Havinga and Veldstra (A) could not corroborate the findings of Riegel and Buchwald, as an extinction of the same order of magnitude as that of p.a.b.a. was found. So these facts will have to be studied more in detail.

Recapitulating, the following conclusions may be drawn from the spectra measured.

1. There is a resemblance in the electronic configuration of p-aminobenzoic acid and of the sulphonamides. They are different from those of compounds like o- and m-aminobenzoic acid and marfanil.
2. In heterocyclic sulphanilamide derivatives like sulphapyridine, sulphathiazole and sulpha-methylpyrimidine a distinct influence of the heterocyclic nucleus upon the sulphanilamide-grouping is perceptible.
3. At p_H 6–7 the amino-group of p-aminobenzoic acid and of the sulphonamides is present but to a small extent as $-NH_3^+$-ion, contrary to those of marfanil (and of m-aminobenzoic acid?).

c. Surface-(interface-)activity (polarographic investigations).

The usual conception about the action of the sulphonamides as antagonists of p-aminobenzoic acid, a substance indispensable to several species of bacteria, is that this substance is regarded as a prosthetic group of an enzyme, which up to now is however, quite unknown.

Though various investigators were able to establish a certain influence of sulphonamides upon enzyme systems, it never has proved to be so essential that the action of the sulphonamides might be explained by it (comp. JULIUS and WINKLER, page 17). This makes it already improbable that the hypothetic p.a.b.a. enzyme system would belong to one of the groups studied up till now.

In view of the fact that the action of the sulphonamides has been associated with oxidation processes in bacteria (FREI and JEZIERSKI, 1943; FREI, 1944) and the possibility of oxidizing p.a.b.a. with phenol oxidase in the presence of catechin and of catalizing the oxidation of p.a.b.a. with H_2O_2 by peroxidase (LIPMANN, 1941), it seemed desirable to obtain supplementary data about the very important group of oxidation-reduction enzymes.

An elegant method of studying oxidation-reduction properties of a compound consists in the polarographic investigation of a solution of this substance, whereby — if reduction, or (with reversed electrodes) oxidation takes place — a characteristic crack in the current-voltage curve is observed, which does not occur in the control (solvent only). (For general data concerning polarography, comp. HEYROVSKY, 1941; KOLTHOFF and LINGANE, 1941).

VELDSTRA (1944) already applied this method to the plant growth substances. In the same way p.a.b.a. was tested, dissolved in 0.01 N HCl and 0.01 N KCl respectively, whereby the range of the voltage amounts to 2 V and 2.8 V resp. on reduction, and to 0.6 V on oxidation. In none of these cases could any essential difference be observed between the controls and the solutions of p.a.b.a. Thus, under these circumstances no reduction or oxidation of p.a.b.a. occurs, which makes a direct function of p.a.b.a. in a redox system unlikely.

As a matter of fact the chemical structure of p.a.b.a. does not point to this either; as MARKEES (1943) expressed it: "It seems to be unable to undergo the reversible intermediate conversions, known in redox systems."

As the action of p.a.b.a. as a "growth substance" for bacteria (lower plants!) bears in a number of points a typical resemblance to that of the growth substances (auxins, heteroauxin and synthetics of analogous action) for higher plants — in both cases overdosage results in inhibition, and the curve, reproducing the activity in its dependence on the p_H of the medium, coincides with the dissociation curve of the acid — VELDSTRA and HAVINGA, in studying the mechanism of action of p.a.b.a., have considered whether this parallelism might have an essential meaning. In that case the possibility is to be faced that p.a.b.a. would act by influencing the permeability of a bacterial membrane, by which the transport of factors indispensable to division might be controlled, as has been made probable for plant growth substances with respect to the protoplasmic membrane and transport of water-soluble nutritious substances (VELDSTRA, 1944). FREI (1944) too, in the investigation already mentioned, has remarked that "besides the displacement theory other chemical and physico-chemical possibilities may be considered, e.g., changes in the permeability and in the swelling of the bacterial ectoplasm."

In view of the quite different structure and configuration of p.a.b.a., its action would have to be of a type, different from that supposed for the plant growth substances, but, like there, it would be localized in a boundary and then, of course, one would have to study boundary models, as was already done with the plant growth substances (VELDSTRA, 1944).

For this purpose VELDSTRA and HAVINGA (A) again utilized the mercury/solution boundary and they determined the boundary activity of p.a.b.a., sulphanilamide and derivatives polarographically, by examining to what extent they suppress, dependent on the concentration, the so-called oxygen maximum in a polarographic curve. The reduction of this maximum comes about by the displacement of oxygen from the air, adsorbed to the cathode, by substances of higher surface activity. The comparative investigation of various compounds can be put into a quantitative form by determining the con-

TABLE III

Investigated compounds	Half suppression value (in micromoles/l = 10^{-6} molar)		
	pH 2	pH 6.2	
		First part of the curve	Second part of the curve
o-Aminobenzoic acid	70		1250
m-Aminobenzoic acid	>10000		1650, 5750
p-Aminobenzoic acid	40		1150
p-Aminobenzoyl-l-glutamic acid	11	120	500
p-Aminobenzoyl-d-glutamic acid	11	—	—
p-Aminobenzoyl-l-aspartic acid	11	96, 240	520
p-Aminophenylacetic acid	6500	2500	
p-Aminomethyl-benzoic acid.HCl	880	250	
1-Aminonaphthalene-4-carbonic acid	11	11	
Sulphanilic acid	>10000	>10000	
Sulphanilamide	150	180	
Sulphanilylguanidine	80	60	
Sulphapyridine	18	29	
Sulphathiazole	17	27	
Sulpha-methylpyrimidine	17	26	
Sulphatetrazole	48		580
(Sulphamethyl)-pyridine	60	110	
Naphthionamide	10	14	
Naphthionylamino-pyridine	8	11	
Naphthionylamino-methyl-pyrimidine	8	10	
p-Aminomethyl-benzene-sulphonamide.HCl (Marfanil)	>5000	190	

centration (expressed in micromoles/l = 10^{-6} molar) by which the oxygen-maximum is suppressed to one half of that of the control (Rayman, 1931). This value, designated as half-suppression-value (H.S.V.) is proportional to the adsorption coefficient of the substance in question (Heyrovsky, p. 171–172) and thus very suited for a quantitative comparison. The determinations were carried out in 0.01 N HCl and in 0.01 N

KCl, in order to establish at the same time the influence of the p_H.

The values obtained are reproduced in Table III.

If, in order to establish the H.S.V., the reduction values found (in mm) are plotted, as a function of the concentration, all the measurings in 0.01 N HCl and the greater part of those in 0.01 N KCl yield a curve, that is to be taken as a normal adsorption curve (see Fig. 24). In 0.01 N KCl solution the curves of the three aminobenzoic acids, p-aminobenzoylglutamic acid, p-aminobenzoylaspartic acid and of sulphatetrazole show a different course, as stated in Fig. 25.

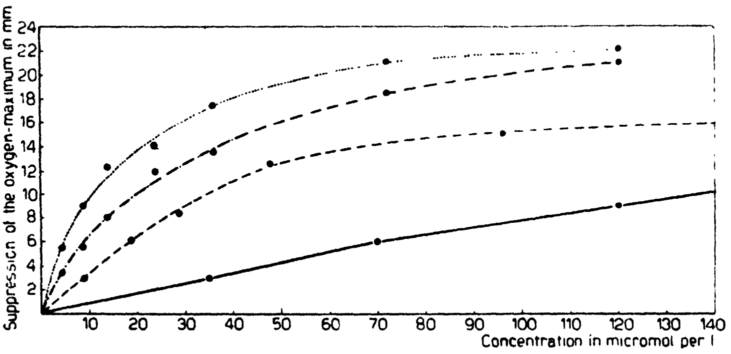


Fig. 24. Suppression of the oxygen-maximum in the polarographic curve by sulphanilamide, p-aminobenzoic acid and their derivatives. In 0,01 N HCl.

———————— sulphanilamide, height of the max. in the control 25 mm

- - - - - - - - sulphanilylguanidine, height of the max. in the control 25 mm

················ sulphapyridine, height of the max. in the control 25 mm

—·— —·— p-aminobenzoic acid, height of the max. in the control 29 mm

On the analogy of the results, obtained by **Frumkin** et al. (1934) in their capillary-electrometric investigations on the adsorption of caproic acid or phenol in the mercury/solution boundary, where the adsorption curves are of the same type, one is inclined to suppose, as was demonstrated there by calculation, that the first part of the curve has reference to a monolayer, the second — ascending — part to polymolecular layers.

However, with the type of compounds investigated, containing basic and acid functions in the same molecule, account has to be taken of the possibility that the orientation in the boundary, changes with the concentration (comp. the scheme, page 95).

For a further discussion of this problem, it is necessary to carry out measurings of boundary potentials. The results of such a research will be communicated elsewhere.

The varying behaviour of p.a.b.a. in a boundary, which depends on the concentration, is also interesting in connection with the fact that p.a.b.a. shows bacteriostatic activity at higher concentrations. If the activity of p.a.b.a. is supposed to unfold itself in a boundary, it is possibly in this way that an explanation can be found for this inversion of the action at higher con-

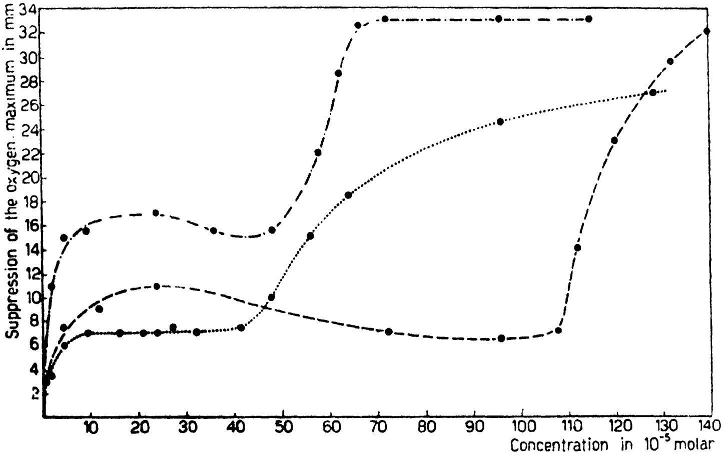


Fig. 25. Suppression of the oxygen-maximum in the polarographic curve by sulphanilamide, p-aminobenzoic acid and their derivatives. In 0,01 N KCl.

·················· sulphatetrazole, height of the maximum in the control of 32 mm

– – – – – – – p-aminobenzoic acid, height of the maximum in the control of 36 mm

—·—·—·— p-aminobenzoyl-l-glutamic acid, height of the maximum in the control of 33 mm centrations. However, it is to be considered, that in the case of the p.a.b.a.-action the rather apolar mercury/solution boundary is a "model" for the physiological boundary to a far less extent as proves to be the case with the plant growth substances, where the interaction with the lipoid membrane is determined chiefly by London–v. d. Waals' forces.

It is interesting that sulphatetrazole (inactive in vivo, very weakly active in vitro), which, as appears from the electrometric titrations, differs far less from p.a.b.a. than the other sulphonamides, shows a polarographic behaviour corresponding with p.a.b.a.

The different course of the curves in the cases described sometimes leads to a "half-suppression" at different concentrations (p-aminobenzoylglutamic acid), or entails that this is only achieved in higher concentrations (in the second part of the curve). For this reason in Table III the measurings in KCl solution are reproduced in two columns.

In comparing the aminobenzoic acids — the measurings in HCl- as well as in KCl-solution — it is striking that p-aminobenzoic acid is the most active and that the meta compound — present in the form of "Zwitterion" to the highest degree — is very weakly adsorbed in the boundary. As compared with p.a.b.a., p-aminophenylacetic acid and p-(aminomethyl)-benzoic acid are adsorbed to a much smaller extent, 1-aminonaphthalene(4)-carbonic acid, however, to a considerably stronger extent.

In connection with the finding of Auhagen (1943), that p-aminobenzoyl-l-glutamic acid as a growth factor (?, comp. p. 31) for Streptobacterium plantarum is ten times as active as p.a.b.a., the stronger surface activity of the glutamic acid derivative, hardly expected with increased solubility in water, seemed very interesting. This parallelism, however, proved to be of no particular interest as p-aminobenzoyl-l-aspartic acid, which, according to Auhagen is inactive, is adsorbed to the same high degree.

Of the chemotherapeutically active sulphonamides, the boundary-activity of sulphanilamide is the lowest, that of sulphanilylguanidine is somewhat stronger, while the heterocyclic derivatives, mutually comparable, are adsorbed to the highest degree. So here is, as with the plant growth substances, a parallelism between boundary-activity and physiological action, as this e.g., finds expression in those low concentrations of the sulphonamides, (determined by Rose and Fox, 1942), only just preventing the growth of a certain inoculation of *Bact. coli* for four days. These values, as compared with the H.S.V. of the compounds in question, are listed in Table IV.

TABLE IV

	Concentration of sulphonamides (in micromoles/l), only just preventing growth of Bact. coli for four days (Rose, Fox)	Half suppression value (in micromoles/l)	
		0.01 N HCl	0.01 N KCl
Sulphanilic acid	Practically inactive	>10000	>10000
Sulphanilamide	2500	150	180
Sulphanilylguanidine	500	80	60
Sulphapyridine	20	18	29
Sulphathiazole	4	17	27

In connection with contemplations on the mechanism of the action of p.a.b.a. and the sulphonamides — see below — we consider the higher surface-activity of the heterocyclic sulphanilamide derivatives to be of the first importance.

The importance of the polarographic investigations consists in the mutual comparison of the sulphonamides or the aminobenzoic acids and related compounds; otherwise, the mercury/solution boundary is perhaps not a suitable model for the p.a.b.a.-sulphonamide action as a whole, to which attention was already drawn. This appears from the fact that the boundary activity of the heterocyclic sulphanilamide derivatives is stronger than that of p.a.b.a., as, on account of the annihilation of the action of a certain sulphonamide concentration by a much lower p.a.b.a. concentration, one would expect the contrary if the ordinary displacement hypothesis is followed.

Our speculations on this subject as given on page 86 ff. may place this question however in another light.

Whatever it may be, from the facts given, one may conclude, as could be expected more or less on account of the structure, that, if the p.a.b.a.-sulphonamide action does take place in a boundary (membrane), it is not due to an interaction with the lipophilic part of a phosphatide constituent (as with the plant growth substances, comp. VELDSTRA, 1944).

For the determination of a possible interaction with the hydrophilic part of it (layer of complex-relations, where electric forces are predominating), more likely for substances of a structure like p.a.b.a. and the sulphonamides, the mercury/solution boundary won't serve the purpose, another type of boundary is required for that (comp. experiments on monolayers, page 85).

The antibacterial activity of naphthionamide and its derivatives proved to be much smaller as we were inclined to expect on account of their marked interface-activity. The behaviour on electrometric titration, which, as compared with sulphanilamide and corresponding derivatives is much the same (see page 67), leads to the conclusion that the very small activity is not to be ascribed to properties completely changed. How far transport difficulties (possibly too strong a non-specific adsorption) play a part, cannot be judged without further measurings. Prolonged investigation of those compounds, which are so closely related to sulphanilamide and its derivatives, may still produce interesting details about the relation between structure and activity.

As appears from the H.S.V. found, the activities of (sulphamethyl)-pyridine and of sulphatetrazole, both lower than that of sulphanilamide, cannot be explained by too small an adsorption. As to sulphatetrazole, some explanation is offered by its abnormal physico-chemical behaviour in electrometric titration and polarographic test, and by its not being adsorbed to Bact. coli, in contrast with the active sulphonamides; for the pyridine derivative these data are not yet complete. These measurements of the adsorption to Bact. coli of the substances studied by us by physico-chemical methods, which were performed following the method of WINKLER and JULIUS with slight modifications, could not be completed owing to war-time difficulties.

With the exception of aminophenylacetic acid, aminomethyl-benzoic acid, sulphanilylguanidine and marfanil, for all the compounds investigated the adsorption at p_H 2 proves to be stronger than at p_H 6.2. The difference, however, is exceptionally large with marfanil, so that here too the characteristic, which marks it off from the sulphonamides, finds expression. The number of available data on polarographic measurings of combinations of p.a.b.a. and sulphonamides is too small yet to discuss them here. The results of further investigations, which very probably will yield interesting facts about the mutual influencing of these compounds in a boundary — important to the discussion on the mechanism of their action — will be reported elsewhere.

The most important results of the polarographic investigations can be summarized as follows:

1. As at voltages up to 0.6 V or 2.8 V no oxidation or reduction of p.a.b.a. takes place, it seems improbable that p.a.b.a. plays a direct part in a redox system as a prosthetic group of an oxidation-reduction enzyme.

2. Ortho-, meta- and para-aminobenzoic acid, two derivatives of the latter and further sulphatetrazole show, dependent on the concentration, a characteristic behaviour in the mercury/solution boundary, whereby, at higher concentrations, account has to be taken of the appearance of polymolecular layers, or perhaps of a different orientation in the boundary. Possibly this offers an explanation for the bacteriostatic properties of higher concentrations of p.a.b.a.

3. From the chemotherapeutically active sulphonamides the degree of activity runs parallel to their boundary-activity, which is considered to be essential, especially for the explanation of the stronger activity of the heterocyclic derivatives of sulphanilamide, as compared with their mother substance.

4. The adsorption of p.a.b.a. in the mercury/solution boundary is stronger than that of sulphanilamide, but less than that of the heterocyclic derivatives. Therefore, this boundary

cannot be considered as a model for the place of action of p.a.b.a. (or the sulphonamides, respectively), following the ordinary displacement theory (compare however page 86 ff.). Moreover, it may be concluded that the p.a.b.a. (sulphanilamide) action is not due to an influencing of the permeability, of the type as is suggested for the plant growth substances.

5. Comparison of the adsorption at p_H 2 or p_H 6.2 respectively, shows that the character of marfanil is different from that of sulphanilamide and its derivatives.

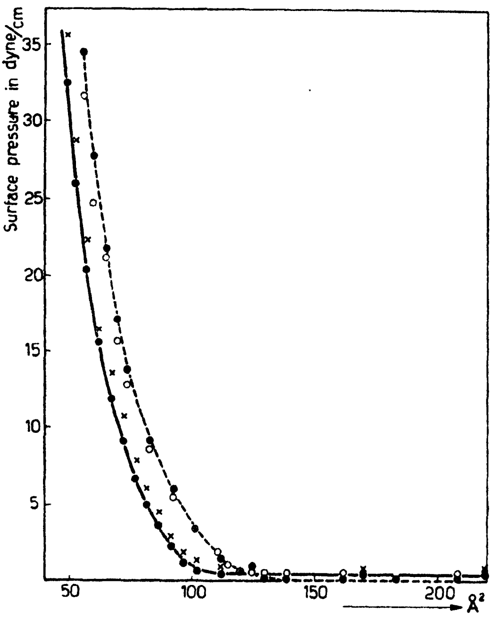


Fig. 26. Interaction of p-aminobenzoic acid and of sulphonamides with monolayers.

—●——●— Lecithin monolayer on 0,04 N sodium acetate buffer, pH 5

× × × Lecithin monolayer on a 10^{-3} molar solution of p.a.b.a in 0,04 N sodium acetate buffer

--●----●-- Lecithin monolayer on 0,02 N. sodium acetate buffer, pH 4

○ ○ ○ Lecithin monolayer on a 3.10^{-3} molar solution of sulphar methyl-pyrimidine-Na in 0,02 N sodium acetate buffe-

d. Experiments on monomolecular layers.

In connection with the polarographic investigations, an examination was made, whether p.a.b.a. and the sulphonamides exert any influence upon another type of boundary, viz., upon a lipoid (lecithin) film, spread on an acetate buffer (Veldstra and Havinga (A)), with which the plant growth substances (e.g., indole(3)acetic acid, naphthalene(1)acetic acid) interact very clearly, as appears from an investigation by Havinga and Veldstra (C).

However, neither p.a.b.a. nor the sulphonamides proved to exercise such an influence upon a monomolecular lecithin layer

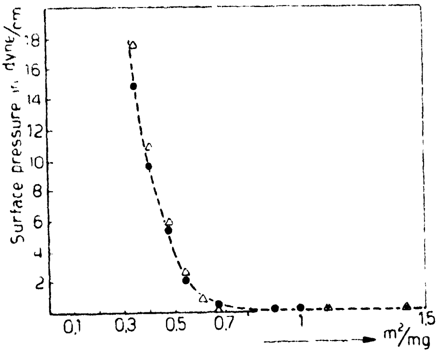


Fig. 27. Interaction of p-aminobenzoic acid and of sulphonamides with monolayers.

--•-----•-- Monolayer of protein from Bact. coli on 0,04 N sodium acetate buffer, pH 5

△ △ △ Monolayer of protein from Bact. coli on a solution of 5.10^{-3} molar p.a.b.a. in 0,04 N sodium acetate buffer

The measurings took place one minute after the addition of the protein solution

As the concentration of the protein solution wasn't exactly known, the abciss-values are but approximate.

(comp. Fig. 26). From this it may be deduced with fair probability that the action of p.a.b.a. or the sulphonamides does not consist in an influencing of the permeability of a bacterial membrane to the effect, mentioned already in the polarographic investigations.

Nor could any effect be observed if p.a.b.a. or sulphonamides were added to the hypophase of monolayers of serum- or bacterial proteins (protein fraction, isolated from Bact. coli), spread on an acetate buffer (Fig. 27).

In connection with views on the mechanism of action of p.a.b.a. (and sulphonamides), to be discussed below, this investigation is continued with films of other compounds (nucleic acids, nucleoproteids), as it seems not out of the question that a specific interaction will occur with a certain type of film, and the discovery of such a relation may result in very important clues to the mechanism of the action.

e. On the mechanism of the action of p.aminobenzoic acid and of the sulphonamides.

Though we can quite agree with the standpoint of the critics (see page 65), holding the view that no exact proof has been furnished as yet for the generally accepted hypothesis that: "the sulphonamides are antagonists of the p.a.b.a.,indispensable for the influenced bacteria", and though not all the facts can be explained on account of this displacement theory, we are, on the other side, convinced that something essential is hidden in the relation p.a.b.a.-sulphonamides.

Apart from the question whether the quintessence of the problem finally will be solved here, it is anyhow a fact, that each solution will have to contain an explanation for the annihilation of sulphonamide-action by a much smaller quantity of the structurally related p.a.b.a.

For this reason the action of p.a.b.a. and of the sulphonamides is treated jointly in the following considerations.

If, with reference to the results of the foregoing physico-chemical investigations, one considers the suppositions, delivered on the mechanism of the p.a.b.a.- or sulphonamide-action respectively, it may be concluded in our opinion, that a function of p.a.b.a. as a prosthetic group, at least for an oxidation-reduction enzyme, is practically out of the question. About other enzyme systems nothing can be said with certainty, as the structure of their prosthetic groups is unknown, and so no reasonable supposition — on account of analogy — can be delivered for p.a.b.a.

As an alternative it has been suggested that not the free p.a.b.a. but a peptide-derivative of it would function as a co-enzyme (Auhagen, 1943), so that p.a.b.a. would be built in the molecule of the co-enzyme. In our opinion an objection may be brought up against this, namely the immediate restoration of division of a culture, inhibited by sulphonamides, by addition of p.a.b.a. (Nitti, Faguet, 1944). From this one gets the impression that p.a.b.a. is really active as such, and thus it may be considered by what means a molecule of relative simple constitution as p.a.b.a. can play such a prominent part.

A possibility like that was presumed by us to lie in a function as a permeability regulator. However, data of polarographic investigations, combined with those obtained with monolayers, preclude this possibility, for a membrane of the phosphatide type anyhow.

For the purpose of a closer examination it is necessary to recapitulate a number of fundamental facts known from literature, as these have to form the base of each well-founded hypothesis.

1. Sulphonamides act in quite a peculiar way, they are slightly or not at all bactericidal, neither are they bacteriostatical in the usual sense, but they prevent division — mostly after a certain lag-time, in which a number of successive divisions of the bacteria have taken place. This effect, however, might be indirect, due to an action on one of the reactions involved in bacterial metabolism (see page 39 ff.).

2. The activity of all sulphonamides is annihilated by p.a.b.a. (in lower concentrations). P.a.b.a. is an antagonist occurring in nature too. The antagonistic action is immediate.

3. For some bacteria p.a.b.a. is a vitamin, that already in an extremely low concentration (10^{-11} g/ml and less) may exert influence upon the multiplication of the bacteria. Probably it is an essential metabolite for many micro-organisms.

Surveying the available data in their entirety one gets from all of them a strong impression that p.a.b.a. plays a part in a mechanism, particularly essential to bacteria in the phase of division. If we define this more precisely we are apt to suppose *that the action takes place in the centre of the cell, from where the chemical occurrences in the cell — especially with regard to cell-division — are regulated.* Thus p.a.b.a. might have a *function either in the formation of the* nucleoproteins, being *at the top of the chemical hierarchy, or, in a determinant phase of the division, interact with* these substances, which, according to CASPERSSON (1938–1940), are essential to this process.

There are indications that the action of the sulphonamides is also related to the mechanism of division. Thus, their action on resting bacteria is practically negligible and moreover bacteria do not multiply under the influence of sulphonamides, but can assume the tenfold of their normal volume in consequence of continued growth (LWOFF et al, 1941). From this, one may conclude that, properly speaking, the drugs don't act as growth- but as division-inhibitors.

Suppositions of ROSE and FOX (1942) in connection with the results of their quantitative analysis of sulphonamide-bacteriostasis are of similar purport. The sulphonamides namely would inhibit the formation of a (hypothetical) division-factor, which is consumed in cell-divison, and p.a.b.a. would in some way be connected with the formation of this factor.

Speculations on a quantitative base on the possible action of p.a.b.a. and the sulphonamides are found in the literature in small numbers only, and mostly they are but little critical, and are even frequently affected with elementary errors.

In order to obtain some idea of the activity of e.g., p.a.b.a., most workers calculate how many molecules of it are available to one bacterium. This is deceptive however, since much the largest part of the p.a.b.a. is to be found in the liquid outside the bacteria, a small part only is taken up in the bacteria themselves.

P.a.b.a. is still active in very large dilutions, thus in the case of Streptobacterium plantarum a concentration of 10^{-11} g/ml or 0.7×10^{-10} molar is sufficient for optimal growth (KUHN, 1942). A solution of 10^{-11} g/ml contains per ml 4×10^{10} molecules of p.a.b.a. or 1 molecule per 2.5×10^{-11} ml. If we take 2×10^{-12} ml as the volume of a bacterium, it follows from these data that on the average only 1 in 10 bacteria is found to contain a molecule of p.a.b.a. Of course, this number is somewhat larger on account of adsorption of the p.a.b.a., but quantitative measurings made in this connection make it probable that this will amount perhaps to a factor of 2 or 3. There is some danger, it is true, in looking into this question from a static point of view only, but on considering it dynamically, it is also difficult to assume a permanent influence of p.a.b.a. upon the occurrences in the cell.

One is inclined to think rather of a "hitting"("Treffer"-)-mechanism, to the effect that each time the p.a.b.a. arrives in the cell at a definite place, where it exercises its action ("receptor", "Haftbezirk"), a reaction takes place or is initiated, which is determinant for the possibility of division. The number of hits, necessary for a division, cannot be stated without a further analysis of experiments on the rate of division as a function of the concentration of p.a.b.a. Probably it is not large and for the sake of concreteness we can assume provisionally the scheme applicable to many biological processes, namely that in a certain phase the presence of one molecule of p.a.b.a. at the place in question in the cell may be determinant as well for the setting-in of a division.

That the places where p.a.b.a. can be bound in such a way that it exerts its influence, are small in number is made probable by the fact that p.a.b.a. already shows optimal activity in extremely low concentrations (see above).

Now one can try to calculate what are the chances that one "receptor" (e.g., a certain position in a boundary) is visited by one molecule of p.a.b.a. in a certain unit of time. There are various methods allowing a certain estimation, though each of them is subjected to great inaccuracy.

In the first place one can hold to the calculations of the number of collisions of molecules in the gas phase. As it is easily perceived the number of collisions per second on a surface enclosed by a circle of diameter σ amounts to:

$$Z_g \sim \tfrac{1}{4}\pi\sigma^2 \cdot \tfrac{1}{6}nv,$$

(n = number of molecules per ml; v = average velocity of the molecules). With $n = 4.10^{10}$, $v = 2.10^4$ cm/sec and $\sigma = 4$ Å $= 4.10^{-8}$ cm, a value of $Z \sim 0.16$ is found.

As to the number of collisions in a liquid phase, on theoretical grounds as well as from measurings of reaction-velocity in bimolecular reactions, it may be assumed that it is of the same order of magnitude as in an equal concentration in the gas phase. (For this and the following speculations see particularly: FOWLER and SLATER (1938); GUGGENHEIM and WEISS (1938); HINSHELWOOD (1938); RABINOWITCH (1938); and further BRADLEY (1937); RABINOWITCH (1937); RABINOWITCH and WOOD (1936)).

However, an alteration occurs then in the periodicity of the collisions. We get series of collisions in rapid succession, designated as "encounters", separated by longer intervals in which no collision takes place. This is intelligible, as on the one hand a molecule in the liquid is moving slower (with a velocity controlled by the diffusion-constant) than in a gaseous phase, on the other hand a molecule, having arrived at a certain place, will stay generally in the vicinity for a considerable time. In ordinary chemical reactions, whereby but one of a large number of collisions is effective, these two factors practically compensate each other. In our problem, however, it is not the number of collisions that is determinant for the "reaction velocity", but the number of encounters of the p.a.b.a. with its receptor. (Only one of the collisions out of the series in an encounter can be a successful hit; a noticeable percentage of the encounters will lead to a positive result). Estimations hereabout give rise to the supposition that the number of encounters is about one hundredth of that of the collisions.

So one arrives at the result that in a molar concentration of 10^{-10}, the average interval between two encounters of p.a.b.a. with the receptor will be of the order of magnitude of ten minutes.

Another method to estimate this very roughly can be based upon the so-called "cage-model" of liquids (ALTAR (1937); LENNARD-JONES and DEVONSHIRE (1937); POLISSAR (1938); TAYLOR (1938); FÜRTH, ORNSTEIN and MILATZ (1939). This image brings the structure of a liquid in close connection with that of a solid, by assuming that a liquid consists of small areas, possessing a regular, nearly crystalline structure. A molecule in the liquid is considered to be in a cage, the walls of which are formed by other molecules of the liquid. In our case one might assume first of all — estimating very roughly — that a part of the receptor forms the base of such a cage.

We imagine that e.g., the amino-group of the p.a.b.a.-molecule has to be situated in this cage, if the molecule should be adsorbed in the right way; moreover it is supposed that, if the amino group arrives in this cage there is a great chance that owing to the specific attraction forces, the rest of the molecule will assume the right position too.

The chance of an amino group being in the cage is $\frac{n}{N}$ (n = molecules of p.a.b.a. per ml, N = total of molecules per ml).

The number of times (Z_d) that a cage changes its "occupant" is connected with the diffusion-constant:

$$D = \frac{(\text{average displacement})^2}{2} \cdot Z_d = \frac{\sigma^2}{6} \cdot Z_d$$

(σ = diameter of the cage)

$$\text{or } Z_d = \frac{6}{\sigma^2} \cdot D.$$

The number of times that an amino-group arrives in the cage is:

$$W = \frac{n}{N} \cdot Z_d = \frac{6n}{N\sigma^2} \cdot D.$$

As in the case of close packing of the molecules $N\sigma^3 = \sqrt{2}$ it follows that:

$$W = \frac{6n\sigma D}{\sqrt{2}} = 33.10^{-3}$$

$$(n = 4.10^{10};\ \sigma = 4.10^{-8}\ \text{cm};\ D = \tfrac{1}{2} \cdot 10^{-5}\ \text{g/sec}).$$

Thus, on an average of once per 30 seconds a cage, which is free in the liquid, will lodge the amino group of a molecule of p.a.b.a.

In our case however the receptor is a cage that is not freely accessible but, owing to the molecules of the "wall" and the layer of watermolecules connected relatively firmly with these, is probably readily accessible from one direction only. On the one hand this is the cause of the fact, that, when once a certain molecule is at the spot, it remains there on the average for a longer time than in a cage being free in the liquid, on the other hand it causes less molecules to arrive there in unit time. Perhaps this may involve a factor of 3–10.

By this picture too we finally arrive at the conclusion that the interval between two encounters will be of the order of magnitude of a few minutes (again with a possible error expressed by a factor 10).

A third method of looking into the question is to consider it as a diffusion problem. This may be done in a classical way, e.g., like v. SMOLUCHOWSKY (1917) did when calculating rapid coagulation of uncharged spherical particles of sols, whereby each collision between two particles leads to an attachment and thus means a "hit". The mathematical handling of a non-sphero-symmetrical problem, as would have to be solved in our case, is difficult, it is true, but if the formulae of v. SMOLUCHOWSKY are applied with some boldness, we arrive again at intervals between two encounters that are of the same order of magnitude as has been found by means of the preceding method.

More recent models of diffusion make use of the "cage" model already mentioned, and also lead to quantitatively comparable results.

So we have to assume that with the present, very dilute, solutions of p.a.b.a. (10^{-11} g/ml) the time that passes on the average before a receptor in the cell is visited by a new molecule of p.a.b.a., will vary from 1 to 100 minutes.

When supposing now — and our picture already implies this more or less — that a proper percentage of the encounters (from 10 to 100 %) will effect a reaction, ending in division of the bacteria, it is easily understood that, as in a p.a.b.a. concentration of 10^{-10} molar the number of favourable collisions for a certain unit of time becomes of the same order of magnitude as the number of divisions, in a molar concentration lower than 10^{-10} the rate of division may be determined by (or be proportional to) the concentration of this "growth substance".

No detailed idea can be formed of the proper action of p.a.b.a., without an investigation directed especially to this.

In connection with its structure and the supposed place of action (centre of the cell, division-mechanism) certain pictures come to the fore indeed, like local electric influencing, building into a "matrix" or rather: structural part of the "nucleus" (nucleoprotein).

It is quite possible that it plays a part in the synthesis of molecules (nucleoproteins), important to the occurrences in the cell, especially to cell-division and that the action would be more or less comparable with the initiation of an autocatalytic process.

Supposed that the sulphonamides act indeed as antagonists of p.a.b.a. (displacement-theory), then, if one asks oneself what they will have to do for inhibiting the interaction of p.a.b.a. with the receptor — necessary to the proceeding of a division — it becomes clear that they will have to "watch" the place in question more or less permanently, in contrast to the p.a.b.a., which has to come there once or a few times only. From this it follows directly:

1. The difference in concentration between p.a.b.a. as a growth factor and the sulphonamides in their action as "anti-division substances". If it were merely the adsorption displacement that came into play, then p.a.b.a. would have to be adsorbed much more strongly than the sulphonamides. In the case of sulphanilamide there are indications for this, but not, however, in the case of its heterocyclic derivatives (compare also the polarographic investigations, page 77, table III).

With the mechanism suggested, this difference in concentration between p.a.b.a. and the sulphonamides is a mere consistency, even if surface activities are comparable!

2. That the "attaching" power (and possibly structural details) may still play an essential part in determining the activity of a sulphonamide.

The lower activity of sulphanilamide, compared with its heterocyclic derivatives, can be explained too from this point of view, and is thus running parallel to its lower surface-activity (see polarographic measurings, page 77, table III).

A somewhat quantitative contemplation on the antagonism p.a.b.a./sulphonamides can take the following form.

In the presence of as little as possible p.a.b.a., sulphonamides (in vitro) act in concentrations of 10^{-5} to 10^{-7} molar. Therefore, with the strongly active sulphonamides the number of molecules per bacterium is not large either: $\sim$ 100 molecules per bacterium. Here too we arrive at the conclusion, that the sulphonamide acts upon one or a few limited places in the cell only. In this case the number of collisions with the receptor is of the order of magnitude of 10^2–10^4 per second (comp. p. 89). As a matter of fact here the calculation becomes still more complicated than with p.a.b.a., as, contrary to the supposition made in that case, a first collision does not have a sufficient effect, and so the "screening-time" has to be calculated, as the same molecule in the same place can be active again and again. This calculation is a perilous task, as we have no sufficient knowledge about the energy by which the molecule is kept at its place. To get an idea we may assume that this energy (v. d. Waals' forces, ionic attraction) in the case of molecules like p.a.b.a. and the simple sulphonamides is practically equal to 10^{-12} erg. The thermal motion has an energy of $kT = 4.10^{-14}$ erg. On account of the thermal motion a molecule vibrates with a frequency of the order of magnitude of 10^{13}/sec (for molecules of not too high a molecular weight; comp. Eyring (1936); Hermans (1939)).

We will make use of the cage-model again to describe the events in the liquid. The molecule jumps from time to time into an adjacent cage. The frequency (Z) of such a jump depends strongly on the activation-energy (E) required: $Z = 2\nu \cdot e^{\frac{-E}{kT}}$. In our case the result is $Z = 2.10^{13} \cdot e^{\frac{-10^{-12}}{4 \cdot 10^{-14}}} = 2.10^{13} \cdot e^{-25} = 10^2 - 10^3$. It is clear that small differences in E bring about a great variation in Z. Nevertheless we should venture to evaluate — also on the grounds of the results of measurements of adsorption — the time of staying as varying from $1 - 10^{-5}$ sec. This makes it intelligible that sulphonamides begin to unfold their activity at molar concentrations of $10^{-5} - 10^{-7}$, as a proper screening can then be expected (number of collisions $\sim 10^3$/sec., staying time $\sim 10^{-3}$ sec.).

If the p.a.b.a., in spite of the watching by the sulphonamide, is to have a proper chance to arrive once at the spot, its concentration has to be higher than in the absence of the sulphonamide. However, in the case of comparable attraction towards the receptor, this concentration can always be considerably lower than that of the sulphonamide, as the p.a.b.a. has not to be constantly present, but will have to break through only once (advantage of the assailant over the defender). So here a kind of displacement equilibrium appears indeed, as it was made probable too by the measurings of Kuhn and coll. (1941).

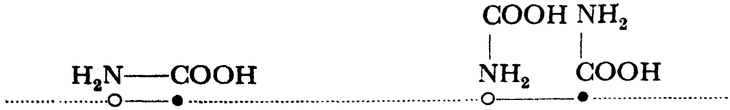
From the point of view developed, the following particulars about the action of p.a.b.a. and the sulphonamides may be considered intelligible now:

1. P.a.b.a. acts instantly, that is to say when it is added to bacteria, which are not in division in the presence of a sulphonamide, the division starts promptly.

2. Of course there are, except for "hits" by p.a.b.a., other mechanisms too that will have to proceed if a division will take place. Before an avalanche occurs, snow will have to fall — for lack of nutritious substance or certain necessary ergons the cell cannot arrive in a state in which division will possibly occur.

In the presence of sufficient p.a.b.a. (more than 10^{-10} molar) the rate of division is determined by these other factors.

3. In higher concentrations ($\sim 10^{-2}$ molar) p.a.b.a. may inhibit growth. Possibly it is the substance itself that blocks the receptor by occupying it too closely, which may be represented schematically as follows:



right filling filled too close

Of course this scheme is completely hypothetical, the action of p.a.b.a. in higher concentrations may just as well be another.

For a different behaviour of p.a.b.a. in a boundary, in a low and in a higher concentration, compare the polarographic measurings (page 78, 79).

4. Each inoculation brings a certain quantity of reserve substances into the medium, owing to which the bacteria, even in the presence of sulphonamides, may carry out a few divisions ("lag-time of action"). If these are consumed, the division comes to a standstill by the sulphonamide blockade and the bacteria die off slowly.

5. It is logical that a specific influence of the sulphonamides upon the ordinary ferment systems (catalase, carbohydrate metabolism, nitrogen metabolism), which perhaps would have made their action comprehensible, could not be demonstrated.

6. If in an experimental way it really appears that some sulphonamides may show growth stimulating activity (LINK, 1943), this might be explained by assuming that they possess only very weak sulphonamide properties, but may liberate p.a.b.a. by displacing it from other places (non-specific adsorption). Possibly some compounds are able to take over the physiological function of p.a.b.a. in a very defective way.

7. Sulphonamides are also active against a few virus diseases (Lymphogranuloma inguinale, Bronchopneumonia (in mice)), and this activity is counteracted by p.a.b.a. (MUDROW and BOCK, 1943). This points to the direction of a possibility of interference with the process of "multiplication" of nucleoproteins!

For the present the subject is treated as simply as possible. We assume that peculiarities, as they present themselves specially with the action of sulphonamides on pneumococci (compare LÄUGER, 1944), where very small structural modifications (e.g., the displacement of a methyl group), cause important changes in their activity, have to be ascribed to secondary influences. In this case we think in the first place of varying permeability through certain bacterial membranes. That in some cases this permeability very probably plays a predominant part in the question of whether a certain compound will be active or not, follows from the fact that in adsorption tests with Bact. coli, contrary to the chemotherapeutically highly active sulphonamides, compounds such as sulphatetrazole sometimes appear to be shut out from the bacteria ("negative adsorption"). (VELDSTRA and HAVINGA, B).

With the view given on the action of p.a.b.a. as a division-regulator and on the possible action of the sulphonamides in this connection, the question: "Do the sulphonamides act by displacement of p.a.b.a.?" is difficult to answer definitely. Starting originally from the supposition that a real adsorption-displacement occurs, we tried to prove this by using HAVINGA's method (1944, see page 137) of determining separately p.a.b.a. and sulphonamide in two liquids obtained in a manner as described schematically below:

1. To 5 ml of a suspension of Bact. coli, 1 ml of a $0{,}5.10^{-3}$ molar solution of sulphapyridine is added and thoroughly mixed. After 30 minutes the bacteria are centrifuged and 3 ml of the clear supernatant liquid are diluted with 1 ml of water.

2. To 5 ml of the same suspension of Bact. coli, 1 ml of water is added. After mixing and waiting for 30 minutes the bacteria are centrifuged. 3 ml of the clear liquid are diluted with 1 ml of a $0{,}25.10^{-3}$ molar solution of sulphapyridine.

It is evident that the measurement of the sulphapyridine content of the liquids 1 and 2 reveals exactly to what extent adsorption has taken place. Comparison of the colour due to p.a.b.a. in the two liquids can give information about the liberation of p.a.b.a. by the treatment of the suspension with the sulphonamide. Evidently in the case of a displacement of p.a.b.a. in series 1 more p.a.b.a. would have to be found. Now, as a matter of fact, in 60 % of the tests of series 1, a coloration appeared indeed in the p.a.b.a. fraction, whereas in series 2 this was never observed. These results are not however sufficiently convincing. Starting from the conception of the action of p.a.b.a. and the sulphonamides given above, on closer examination one must conclude moreover that the specifically adsorbed p.a.b.a. (physiologically "acting") cannot be determined in consequence of too low a concentration and the coloration observed in series 1 would have to be ascribed to the liberation of unspecifically adsorbed p.a.b.a. In that case an exact proof is not to be furnished in this way.

For a further development of the ideas first of all materials are to be expected from model-tests, as e.g., the interaction with monolayers of nucleic acids and related compounds. These investigations are in progress.

Summary.

1. On account of the results of the physico-chemical investigations the action of p.a.b.a. (or of a derivative) as a prosthetic grouping of an oxidation-reduction enzyme is considered to be improbable. Its function as a permeability-regulator can with a fair probability be excluded too.

2. The hypothesis that p.a.b.a. acts (indirectly?) as a regulator of division is brought to the fore. It is considered possible that p.a.b.a. has an influence on the synthesis of nucleoproteins, or interacts — in a determinant phase of the division — with these compounds, essential to this process.

3. From data of the literature it may be deduced that the sulphonamides too interfere with the mechanism of division rather than that of growth.

4. On account of a quantitative contemplation, the action of p.a.b.a. is considered to be possibly a hitting ("Treffer")-mechanism, and it is calculated that at the just fully active dilution of p.a.b.a. (10^{-11} g/ml) the order of magnitude of the average time between two hits on a certain place of action is 1–100 minutes. Then at a concentration of p.a.b.a. $< 10^{-10}$ molar the rate of division will be determined by (or be proportional to) the concentration of this factor, as the number of encounters with the receptor becomes of the same order of magnitude as the number of divisions.

5. From this conception it follows quite logically that the action of the sulphonamides, if considered as antagonists of p.a.b.a., even in the case of comparable surface activity, is efficient only in concentrations higher than that of p.a.b.a. The sulphonamides have to "watch" the p.a.b.a.-receptor permanently, whereas p.a.b.a. for the initiation of a division has

to come there once or a few times only. A semi-quantitative calculation for this case shows that this may occur with the concentrations of the sulphonamides known to be only just active (10^{-5}–10^{-7} molar, depending on the surface activity of the compound). On account of this conception several peculiarities of the action of p.a.b.a. and of the sulphonamides may be considered intelligible.

LITERATURE

Cited on page

ALBERT, A. AND GOLDACRE, R., *Relative acidity and basicity of sulphanilamide and of p-aminobenzoic acid.* Nature, 149 (1942) 245. C (1942) II, 804 68

ALTAR, W., *A study of the liquid state.* J. Chem. Physics, 5 (1937) 577 90

AUHAGEN, E., *p-Aminobenzoylglutaminsäure, ein gegen Sulfonamide wirksameres Derivat des Vitamin H'.* H. S. Zeitschr. physiol. Chem., 277 (1943) 197 . . . . 80, 87

BELL AND ROBLIN, *J. Amer. Chem. Soc.*, 64 (1942) 2905. Cited from IVANOVICS (1944) 69

BLANCHARD, K. C., *J. Biol. Chem.*, 140 (1941) 919. Cited from MARKEES (1943) 65

BRADLEY, R. S., *The rate of unimolecular and bimolecular reactions in solution as deduced from a kinetic theory of liquids.* Trans. Faraday Soc., 33 (1937) 1185 90

CASPERSSON, T. AND SCHULTZ, J., *Nucleic acid metabolism of the chromosomes in relation to gene reproduction.* Nature, 142 (1938) 294 88

CASPERSSON, T. AND SCHULTZ, J., *Pentose nucleotides in the cytoplasm of growing tissues.* Nature, 143 (1939) 602 88

CASPERSSON, T., *Über die Rolle der Desoxyribonukleinsäure bei der Zellteilung.* Chromosoma, 1 (1939—1940) 147 88

CASPERSSON, T., *Die Eiweissverteilung in den Strukturen des Zellkerns.* Chromosoma, 1 (1939—1940) 562 88

CASPERSSON, T., *Nukleinsäureketten und Genvermehrung.* Chromosoma, 1 (1939—1940) 605 88

DAVIS, B. D., *Binding of sulphonamides by plasm-proteins.* Science, 95 (1942) 78. C (1942) II, 683 69

DAVIS, B. D. AND WOOD, W. B., *Proc. Soc. exp. Biol. Med.*, 51 (1942) 283. Cited from IVANOVICS (1944) 69

DEDE, L. AND ROSENBERG, A., *Über innere Molekülverbindungen.* Ber., 67 (1934) 147 . . . . 71, 73

DEVOTO, G., *Anwendung der Dielektrizitätskonstanten-Messungen in wässriger Lösung auf dem Gebiet der organischen Chemie.* Z. f. Elektrochemie, 40 (1934) 490 74

Cited on page

Eyring, H., *Viscosity, plasticity and diffusion as examples of absolute reaction rates.* J. chem. Physics, 4 (1936) 283 94

Fildes, P., *The mechanism of the anti-bacterial action of mercury.* Brit. J. exp. Path., 21 (1940) 67 65

Fowler, R. H. and Slater, N. B., *Collision numbers in solutions.* Trans. Faraday Soc., 34 (1938) 81 90

Frei, W., *Oxydationsvorgänge bei Bakterien und ihre Bedeutung für Pathogenese und Chemotherapie.* Schweiz. Med. Wochenschr., 74 (1944) 69 . . . . 75, 76

Frei, W. and Jeziersky, A., *Über die Beeinflüssung der bakteriellen Dehydrierung durch Sulfanilamide.* Schweiz. Med. Wochenschr., 73 (1943) 754 75

Frumkin, A., Gorodetzkaja, A. and Tschugunoff, P., *Über die Bildung von polymolekularen Schichten an der Trennungsfläche Quecksilber/Lösung.* Acta Physicochimica U.R.S.S., 1 (1934) 12 . . 78

Fürth, R., Ornstein, L. and Milatz, J. M. W., *Zur Theorie der Flüssigkeiten.* Proc. Acad. Wetensch. Amsterdam, 42 (1939) 107 90

Guggenheim, E. A. and Weiss, J., *The application of equilibrium theory to reaction kinetics.* Trans. Faraday Soc., 34 (1938) 57. . . 90

Halbedel, G., *Messmethoden und Messungen der Dielektrizitätskonstante amphoterer Elektrolyte.* Z. f. physik. Chem., 33 B (1936) 83 . . 74

Havinga, E., *Die Bestimmung der p.Aminobenzoesäure und des Sulfapyridins, bzw. ähnlicher Verbindungen nebeneinander.* Rec. trav. chim., 63 (1944) 243 97

Havinga, E. and Veldstra, H., *Researches on sulphanilamide-derivatives IV. Ultraviolet absorption spectra and electrometric titrations.* To be published in Rec. trav. chim. (A) 65, 70, 74

Havinga, E. and Veldstra, H., *Researches on sulphanilamide derivatives VI. On the mechanism of the action of p-aminobenzoic acid and of the sulphonamides.* To be published in Rec. trav. chim. (B) 65, 88ff

Havinga, E. and Veldstra, H., *Researches on plant growth substances XIV. Interaction of synthetic growth substances with lipophylic monolayers.* To be published in Rec. trav. chim. (C.) 85

Hedestrand, G., *Über die Dielektrizitätskonstanten wässriger Lösungen einiger Aminosäuren.* Z. physik. Chem., 135 A (1928) 36 74

Hermans, J. J., *The application of the cage theory of liquids to the problem of ion mobilities.* Rec. trav. chim., 58 (1939) 917 94

Heyrovsky, J., *Polarographie,* Wien, 1941 . . . . 75, 77

Hinshelwood, C. N., *The present position of the theory of reaction velocity.* Trans. Faraday Soc., 34 (1938) 105 90

Ivánovics, G., *Experimentelle Chemotherapie der bakteriellen Infektionen.* Acta Medica Szeged, Tom. XII Fasc. 1. (1944) 69

Jensen, K. A., Schmith, K. and Brand, P., *Über die bakteriostatische Wirkung von p-Aminomethyl-benzolsulfonamid.* Klin. Wochenschr., 21 (1942) 1042 69

C
on

JENSEN, K. A. AND FRIEDIGER, A., *Sulfanilylderivater af heterocykliske aminer IX. Dipolmomenter af sulfanilamid derivater.* Dansk Tidsskr. Farmac., 16 (1942) 280 68

JENSEN, K. A. AND SCHMITH, K., *Zusammenhänge zwischen chemischer Konstitution und bakteriostatischer Wirkungen bei Sulfonamiden.* Z. f. Immunitätsforsch., 102 (1942) 261; 105 (1944) 40 69

JULIUS, H. W. AND WINKLER, K. C., *On the action of sulfanilamide VIII. The mechanism of its action.* Ant. v. Leeuwenhoek, 8 (1942) 139 65

KIMMIG, J. AND WESELMAN, H., *Die Bindungsverhältnisse zwischen Bluteiweisskörper und Sulfonamiden.* Arch. Dermatologie Syphilis, 182 (1941) 436 69

KLOTZ, J. M. *Science*, 98 (1943) 60. Cited from IVANOVICS (1944) 69

KOLTHOFF, I. M. AND LINGANE, J. J., *Polarography.* (Polarographic Analysis and Voltametry, Amperometric Titrations.) New York, 1941 75

KUHN, R., *Vitamine und Arzneimittel.* Die Chemie, 55 (1942) 1 . . . 88

KUHN, R. AND SCHWARZ, K., *Solierung des Wuchsstoffes H' aus Hefe.* Ber., 74 (1941) 1617 65

KUHN, R., WIELAND, TH. and MÖLLER, E. F., *Synthese des (α,γ-Dioxydimethyl-butyryl)-taurins, eines spezifischen Hemmstoffes für Milchsäurebakterien.* Ber., 74 (1941) 1605 95

LENNARD-JONES, J. E. AND DEVONSHIRE, A. F., *Critical phenomena in gases I.* Proc. Roy. Soc. A. 163 (1937) 53 90

LÄUGER, P., SUTER, R. and MARTIN, H., *Zusammenhänge zwischen Konstitution und Wirkung bei Sulfonamiden.* Z. f. Immunitätsforsch., 105 (1944) 78 . . . . 65, 96

LINK, TH., *Über die Chemoresistenz von Staphylokokken gegenüber Sulfonamiden.* Z. f. Immunitätsforsch., 104 (1943) 441 96

LIPMANN, F., *Catalysis of the oxidation of p.aminobenzoic acid by peroxidase and its inhibition by sulfanilamide.* J. Biol. Chem., 139 (1941) 977. C (1943) II, 1555 75

LWOFF, A., NITTI, F., TRÉFOUËL, J. AND HAMON, V., *Recherches sur le sulfamide et les antisulfamides.* Ann. Inst. Pasteur, 67 (1941) 9 88

MARKEES, S., *Über p.Aminobenzoesäure, das sogenannte Vitamin H'.* Schweiz. Med. Wochenschr., 73 (1943) 1479 . . . . 65, 75

MUDROW, L. AND BOCK, M., *Der Antagonismus von Sulfonamiden und Para-Aminobenzoesäure bei experimentellen Spirochäten-, Spirillen- und Virusinfektionen.* Z. f. Immunitätsforsch., 104 (1943) 463 96

NITTI, F. AND FAGUET, M., *Sur le temps d'action de certaines substances antagonistes.* Ann. Inst. Pasteur, 70 (1944) 124 87

NORTHEY, E. H., *Structure and chemotherapeutic activities of sulfanilamide derivatives.* Chem. Rev., 27 (1940) 85 69

PESTEMER, M. AND FLASCHKA, H., *Über den Einflusz der Stellung von Substituenten auf die Ultraviolettabsorption des Benzolchromophors.* Mh. Chem., 71 (1938) 325 74

Cited on page

Polissar, M. J., *A kinetic approach to the theory of conductance of infinitely dilute solutions based on the "cage" model of liquids.* J. Chem. Physics 6 (1938) 833 90

Rabinowitch, E., *Collision, coordination, diffusion and reaction velocity in condensed systems.* Trans. Faraday Soc., 33 (1937) 1225 90

Rabinowitch, E., *Discussion remarks. General Discussion "Reaction Kinetics".* Trans. Faraday Soc., 34 (1938) 113, 121 90

Rabinowitch, E. and Wood, W. C., *The collision mechanism and the primary photochemical process in solutions.* Trans. Faraday Soc., 32 (1936) 1381 90

Rayman, B., *Polarographic studies with the dropping mercury Kathode XIX. The effect of some organic dyes on the maximum of current due to electroreduction of oxygen.* Collection Czechoslovak Chem. Commun. 3 (1931) 314 77

Riegel, E. R. and Buchwald, K. W., *Ultraviolet absorption of para-aminobenzoic esters in water solution.* J. Amer. Chem. Soc., 51 (1929) 484 . . . . 73, 74

Rose, H. M. and Fox, Ch. L., *A quantitative analysis of sulphonamide bacteriostasis.* Science, 95 (1942) 412. C (1943) I 536 . 81, 88

Rubbo, S. D. and Gillespie, J. M., *Para-aminobenzoic acid as bacterial growth factor.* Nature, 146 (1940) 838 65

Rune Frisk, A., *Sulfanilamide derivatives. Chemotherapeutic evaluation of N^1-substituted sulfanilamides.* Stockholm, 1943 and Acta Medica Scand. Suppl. CXLII, 1943 69

Smoluchowsky, M. von, *Versuch einer mathematischen Theorie der Koagulationskinetik kolloider Lösungen.* Z. physik. Chem., 92 (1917) 129 92

Taylor, H. S., *The temperature variation of diffusion processes.* J. Chem. Physics, 6 (1938) 331 90

Veldstra, H., *Nieuwe vooruitzichten voor de chemotherapie van bacteriële infectieziekten.* Chem. Weekblad, 39 (1942) 506 65

Veldstra, H., *Researches on plant growth substances IV. Relation between chemical structure and physiological activity I.* Enzymologia XI, (1944) 97 . . . . 75, 76, 82

Veldstra, H., *Researches on plant growth substances V. Relation between chemical structure and physiological activity II. Contemplations on place and mechanism of the action of the growth substances.* Enzymologia XI, (1944) 137 . . . . 75, 76, 82

Compare this Series: Koningsberger, van der Lek and Veldstra, *Investigations on phytohormones and their practical applications.* Amsterdam 1945

Veldstra, H., and Havinga, E., *Researches on sulphanilamide-derivatives III. The behaviour of sulphanilamide derivatives with respect to the mercury/solution interface (polarographic investigations) and to lecithin- and protein-monolayers.* To be published in Rec. trav. chim. (A). 65, 76, 85

Cited
on page

VELDSTRA, H. and HAVINGA, E., *Researches on sulphanilamide derivatives V. Adsorption and mutual displacement of p-aminobenzoic acid and of some sulphanilamide derivatives on Bact. coli.* To be published in Rec. trav. chim. (B) 65, 97

WINKLER, K. C. AND JULIUS, H. W., *Über den Wirkungsmechansismu der Sulfonamide XI. Über den Wirkungsmechanismus des p-Amino-methyl-benzolsulfonamids (Marfanil).* Schweiz. Zeitschr. f. Pathol. u. Bakteriol. VI (1944) 455 69

WOODS, D. D., *The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide.* Brit. J. exp. Path., 21 (1940) 74
Compare WOODS, D. D. AND FILDES, P., *Chem. Ind.*, 59 (1940) 133 65

III. CHEMICAL INVESTIGATIONS

§ I. SYNTHESIS AND ACTIVITY OF SULPHANILAMIDE-DERIVATIVES AND RELATED COMPOUNDS

After it had become clear from researches in the domain of the sulphonamides that the derivatives, obtained by introduction of the heterocyclic pyridine- resp. thiazole-nucleus into sulphanilamide (sulphapyridine and sulphathiazole), are superior to the mother substance, a number of investigators in the Netherlands studied the question whether this effect could also be obtained by means of other heterocycli.

Backer et al. (1941–1944; A) especially synthesized for this purpose a larger number of sulphanilamide derivatives in which one hydrogen atom of the SO_2NH_2-grouping was replaced by the pyrimidine-, thiazole-, selenazole-, oxazole- or isoxazole-nucleus, resp. by derivatives of them. The activity of the compounds obtained was tested in vivo on mice infected with pneumococci (of type I) and the progress of the infection checked by counting bacteria in the blood twice a day by the plate method. The activity was compared with that of sulphapyridine.

In a number of cases the action was also studied in vitro, by determining the time required for a culture of pneumococci in ascites-broth to become turbid, in the presence of the substance to be examined (in varying concentrations), compared with a control.

Veldstra et al. (1942, 1943, A) carried out similar experiments with a double purpose:

1. To find new compounds of chemotherapeutic value and if possible of smaller toxicity and greater solubility than those of sulphapyridine, sulphathiazole etc.
2. To collect more data on the connection between chemical structure and activity.

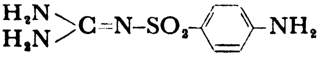
In connection with the latter the research included, besides heterocyclic sulphanilamides, various other compounds. The activity was examined:

a. In vivo in mice, by determination of the average surviving time in a group after ten days, after infection with pneumococci (type I) or streptococci (S. pyogenes Rosenbach) and daily oral administration of the substance (3×10^{-5} moles).

b. In vitro in streptococci according to the counting method of Julius (1938). This method has a great advantage over measuring of the turbidity, not only by allowing a more strict differentiation, but also by procuring insight into the type of the action, as the progress can be reproduced in a typical curve which can be compared with that of a control or sulphanilamide.

a. Derivatives of guanidine.

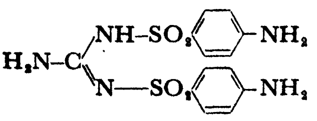
Marshall et al. (1940) obtained sulphanilylguanidine[1] as early as 1940. From that time it was examined at large clinically in the U.S.A. and in Switzerland (comp. Marshall, 1941; Rossi, 1944) and it seems to be of particular significance to intestinal infections, probably because of its very low resorption from the intestine.



Veldstra et al. (A) also prepared this compound, for the purpose of making possible clinical examination in the Netherlands (for the results obtained with "Sulfentidine" compare Chapter IV, page 150).

Tested in vivo with streptococci sulphanilylguanidine is but faintly active, in vitro however it surpasses the activity of sulphanilamide.

Backer and Moed (A) examined disulphanilylguanidine. Tested in vitro with pneumococci the substance is equivalent to sulphanilamide, but in vivo it is inactive. The action upon dysentery bacilli (Shiga-Kruse, Flexner, Kruse-Sonne, Newcastle) in



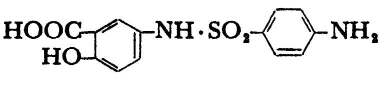
---

[1] The name "sulphaguanidine", which is also used, ought to be cancelled, as "sulpha" indicates the sulphanilylamino-grouping and thus sulphaguanidine refers to a derivative of aminoguanidine.

vitro is very weak and in vivo too it is much smaller than that of sulphanilylguanidine.

b. Derivatives of salicylic acid.

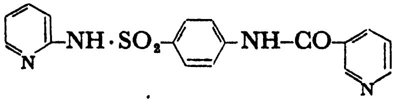
On the assumption that a combined action of sulphanilamide and salicylic acid might result from it, Veldstra and coworkers (A) prepared 5-sulphanilylamino salicylic acid:



In vivo, with streptococci, the substance is practically inactive, it delays the death of the animals only to a small extent. In vitro the activity is comparable with that of sulphanilamide.

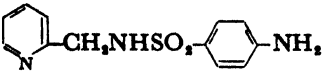
c. Derivatives of pyridine.

As it is well-known that certain undesired secondary effects, appearing in the sulphonamide therapy, are favourably influenced by nicotinic acid or its amide, N^4-nicotinyl-sulphapyridine was synthesized (Veldstra and Wiardi, 1942):



This substance did not yield valuable results, as in vivo the activity against pneumococci had disappeared and that against streptococci is merely comparable with that of sulphanilamide. In vitro (streptococci) the substance possesses but a small activity (Fig. 28).

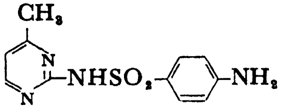
A substance, interesting from a theoretical point of view too, was obtained by reaction of picolylamine with p-acetylaminobenzenesulphonyl chloride and saponification of the acetyl compound formed. This "homologue" of sulphapyridine, namely 2-(sulphanilylaminomethyl)-pyridine proved to be



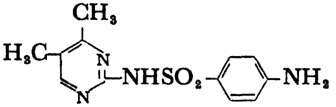
inactive against pneumococci and streptococci when tested in vivo; in vitro (streptococci) some activity is perceptible, but markedly smaller than that of sulphanilamide (Fig. 29).

d. Derivatives of pyrimidine.

BACKER and GREVENSTUK (1941 b; 1942 a; 1945) prepared 18 derivatives of pyrimidine, six of which:



2-sulphanilylamino-
4-methyl-pyrimidine



2-sulphanilylamino-
4,5-dimethyl-pyrimidine

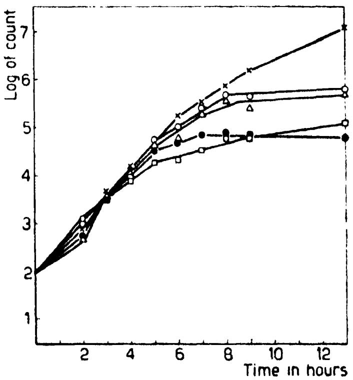


Fig. 28

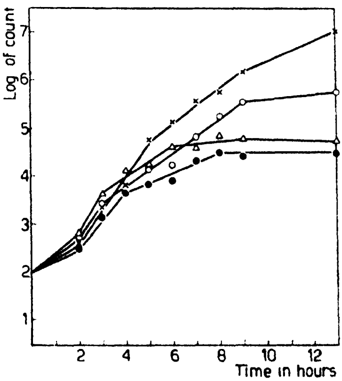


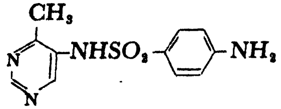
Fig. 29

Fig. 28. Growth of S.pyogenus Ros. in culture media containing sulphonamides.

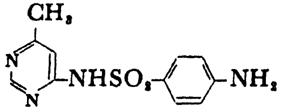
×——×	Control	
●——●	Sulphanilamide	120.10^{-5} mol/l
○——○	N^4-Nicotinyl-sulphapyridine	60.10^{-5} mol/l
△——△	N^4-Nicotinyl-sulpha-4-methylpyrimidine	60.10^{-5} mol/l
□——□	N^4-Nicotinyl-sulphathiazole	60.10^{-5} mol/l

Fig. 29. Growth of S.pyogenus Ros. in culture media containing sulphonamides.

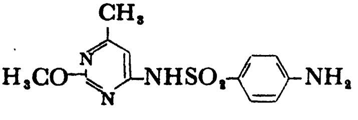
×——×	Control	
●——●	Sulphanilamide	60.10^{-5} mol/l
○——○	2-(Sulphamethyl-)pyridine	60.10^{-5} mol/l
△——△	5-Sulphatetrazole	60.10^{-5} mol/l



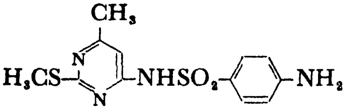
5-sulphanilylamino-
4-methyl-pyrimidine



6-sulphanilylamino-
4-methyl-pyrimidine



6-sulphanilylamino-
2-methoxy-4-methylpyrimidine



6-sulphanilylamino-
2-methylthio-4-methyl-pyrimidine

proved to possess an activity equal to or perhaps even stronger than that of sulphapyridine (in vivo, against pneumococci).

It is remarkable that with the 4-methylpyrimidines the place of the sulphanilylamino group has no influence upon the activity, while in the case of the unmethylated pyrimidine this influence is very strong: 2-sulphanilylamino-pyrimidine is very active, 6-sulphanilylaminopyrimidine on the contrary is quite inactive (ROBLIN, 1940). In the latter compound the introduction of a methyl-group in position 4 thus causes a powerful increase of the activity; the same effect takes place in the change of 6-sulphanilylamino-2-methoxypyrimidine (inactive) into 6-sulphanilylamino-2-methoxy-4-methylpyrimidine (highly active).

The fact that similar transformations do not take place (or to a less extent) with derivatives of 2-sulphanilylamino- or 5-sulphanilylamino-pyrimidines, and the introduction of a methyl-group in position 5 has no comparable effect either, shows the selective mutual influencing of the substituents.

About the actual meaning of this, nothing can as yet be said with any certainty. On the whole substitutions in sulphanilamide-derivatives may lead to great surprises (comp. LÄUGER, 1943).

Except for the 6-sulphanilylaminopyrimidines, the introduction of a OCH_3-group in the pyrimidine-nucleus generally proves to decrease activity and to increase toxicity. On substitutions with SCH_3, this occurs to a less extent.

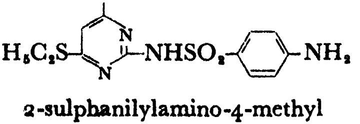
Introduction of hydroxyl-groups in 5-sulphanilylaminopyrimidines and of phenylthio- or anilido-groups in 2-sulphanilylaminopyrimidines leads to decreased therapeutic activity in all these cases. So, with exception of the introduction of a methyl-group in position 4, all kinds of substitutions in the pyrimidine nucleus produce a diminished activity.

Veldstra and Wiardi (1942) prepared 2-sulphanilylamino-4-methylpyrimidine, also obtained by Backer and Grevenstuk in a different way, and affirmed its strong action against pneumococci and streptococci. As pharmacological examinations revealed its comparatively low toxicity and moreover the solubility of its acetylderivative proved to be larger than that of acetylsulphapyridine, the compound was subjected to an ample clinical investigation. The results of this were so auspicious (comp. Chapter IV, page 149) that preparation on a technical scale was started and the substance was put on the market (Percoccide = sulpha-methylpyrimidine).

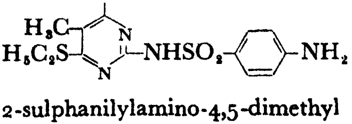
2-Sulphanilylamino-4-n-propylpyrimidine was synthesized as well, but it proved most remarkably to be quite inactive against pneumococci in vivo, while its action against streptococci is identical with that of the methyl derivative. In vitro (streptococci) the activity is almost equal to that of sulphanilamide. On the whole one may conclude from data in literature that, in this respect, pneumococci are much more "sensitive" towards changes in chemical structure than streptococci; the reason for which is quite unknown.

Just as with the corresponding sulphapyridine, the activity of N^4-nicotinyl-sulphamethylpyrimidine on streptococci in vivo was nearly equivalent to that of sulphanilamide; the activity in vitro was much smaller (Fig. 28).

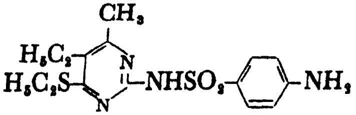
Veldstra et al. (A) further obtained three poly-substituted pyrimidines, viz.:



2-sulphanilylamino-4-methyl 6-ethylthiopyrimidine



2-sulphanilylamino-4,5-dimethyl 6-ethylthiopyrimidine



2-sulphanilylamino-4-methyl-
5-ethyl-6-ethylthiopyrimidine

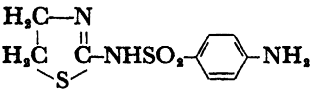
Introduction of the ethylthio group, as compared with sulphamethylpyrimidine, causes a somewhat lessened activity against streptococci in vivo, which diminishes to about one half in the sequence given. In vitro the mutual relations are the same, the activity however is but small.

e. Derivatives of thiazole.

Their preparation and testing on pneumococci was carried out by Backer and De Jonge (1941a, 1942b, 1942). Equivalent to the activity of 2-sulphathiazole is that of its 4-methyl-derivative. On substitution with more, or larger, alkyl-, or aryl-groups however, the activity is lowered and toxicity is increased.

Carboxy-derivatives do not show any activity; on simultaneous substitution with methyl in position 4 however, a substance of proper activity is obtained (2-sulpha-4-methyl-5-carboxy-thiazole).

2-Sulphanilylamino-Δ^2-thiazoline:



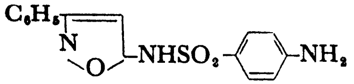
may be compared as to its activity with sulphapyridine.

For the same reason as in the case of nicotinyl-sulphapyridine Veldstra et al. (A) prepared N^4-nicotinylsulphathiazole, which developed an action against streptococci both in vivo and in vitro, equal to that of sulphanilamide (Fig. 28).

f. Pyrazole-, selenazole-, oxazole- and isoxazole-derivatives.

4-Sulphanilylamino-5(3′-pyridyl)pyrazole, obtained by Veldstra and Wiardi (1942) proved to be inactive, when tested in vivo on pneumococci and streptococci. The same can be said, with regard to pneumococci, of the compounds made by **Backer** and **de Jonge** (1941a, 1942b, 1942): 2-sulphanilylamino4-phenylselenazole and 2-sulphanilylamino-5-phenyloxazole.

While 5-sulphanilylamino-3-methylisoxazole is but faintly active, the corresponding 3-phenyl derivative:



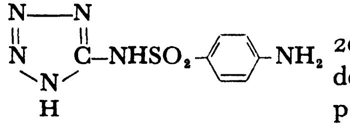
has a striking resemblance to sulphapyridine.

2-Sulphanilylamino-4-methylselenazole, though appreciably active is more toxic than the corresponding thiazole.

g. Triazole- and tetrazole-derivatives.

In this series **Backer** and **de Jonge** (1943a, 1942) prepared 5-sulphanilylamino-3-methyltriazole, N-sulphanilyl-3-methyltriazolone-imide-5 and N-sulphanilyl-3-triazolone-imide-5. These compounds had no action upon pneumococci in vivo.

For the purpose of studying the influence of an acid heterocyclic nucleus and supposing at the same time the possibility of getting at a compound of larger solubility, **Veldstra** and **Wiardi** (1942) synthesized 5-sulphanilylamino-tetrazole:



The desired substance (m.p. 202° C) was obtained by condensing 5-aminotetrazole with p-acetylaminobenzene-sulphonyl chloride in an aqueous, alkaline medium and saponification of the acetyl derivative (m.p. 207° C, first report 202°C) with n. NaOH. Indeed the substance proved to be much more soluble than e.g., sulphapyridine and sulphathiazole, but in vivo it did not show any activity against pneumo- and streptococci. In vitro on streptococci the substance is somewhat less effective than sulphanilamide. (Fig. 29).

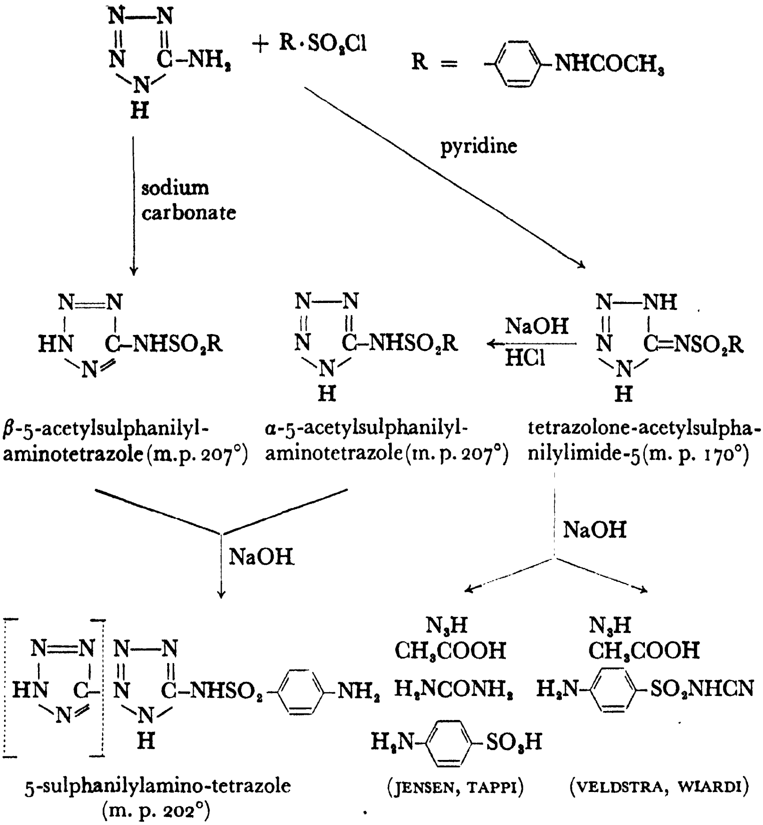
Neither **Roblin** et al. (1940) nor **Jensen** (1941b) have succeeded in preparing this compound, as the former authors, on reduction of 5-p.nitrobenzene-sulphonamidotetrazole, obtained nothing but sulphanilylguanidine, and the acetyl-compound (m.p. 170° C), prepared by **Jensen** by condensation in pyridine solution, could not be deacetylated without scission into azoimide, sulphanilic acid, acetic acid and urea.

Tappi and Migliardi (1941) described the same acetyl compound as Jensen and stated to have obtained 5-sulphanilylaminotetrazole (m.p. 290° C) by saponification in an acid medium. A renewed investigation by Tappi (1943) led to the confirmation of the objection of Veldstra and Wiardi (1942) that by the acid saponification no 5-sulphanilylaminotetrazole would have been formed. Tappi now obtained quite the same results as Jensen.

Jensen and Rosenlund Hansen (1943), on the ground of data from earlier experiments, arrived at the conclusion that the acetylcompound of m.p. 202° C, obtained by Veldstra and Wiardi, was not 5-acetylsulphanilylaminotetrazole but an x-acetylsulphanilyl-5-aminotetrazole. They did not succeed in deacetylating this substance without a rather thorough decomposition.

Veldstra and Wiardi (1943), however, were able to prove, also on the score of electrometric titrations and comparison of the UV-absorption spectra, that in the reaction of 5-aminotetrazole and pacetylaminobenzenesulphonyl chloride in an aqueous medium, 5-acetylsulphanilylaminotetrazole is formed indeed, and from this by saponification 5-sulphanilylaminotetrazole. The acetylcompound of m.p. 170° C, prepared by Tappi and by Jensen by carrying out the reaction in pyridine, is tetrazolone-acetylsulphanilylimide-5, a substance which cannot be deacetylated without decomposition. If this acetylcompound is dissolved in sodium hydroxide and precipitated with hydrochloric acid, another acetylcompound is formed (m.p. 207° C), which, judging from the type of its crystals and a different conduct on electrometric titration,is in all probability different from the 5-acetylsulphanilylaminotetrazole (m.p. 207° C) of Veldstra and Wiardi. Both substances of m.p. 207° C yield the same 5-sulphanilylaminotetrazole on deacetylation.

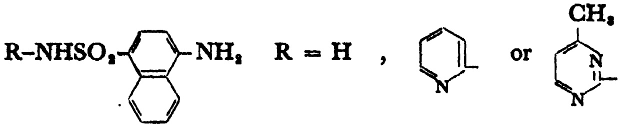
In all likelihood the mutual relations are as follows:


+ R·SO₂Cl
R = –C₆H₄–NHCOCH₃
sodium carbonate
pyridine
NaOH
HCl
β-5-acetylsulphanilylaminotetrazole (m.p. 207°)
α-5-acetylsulphanilylaminotetrazole (m.p. 207°)
tetrazolone-acetylsulphanilylimide-5 (m. p. 170°)
NaOH
NaOH
5-sulphanilylamino-tetrazole (m. p. 202°)
N₃H
CH₃COOH
H₂NCONH₂
H₂N–C₆H₄–SO₃H
(JENSEN, TAPPI)
N₃H
CH₃COOH
H₂N–C₆H₄–SO₂NHCN
(VELDSTRA, WIARDI)

h. Derivatives of naphthionamide.

As it had appeared from polarographic analysis (comp. p. 77, table III) that the strongly active heterocyclic sulphanilamide-derivatives, as compared with sulphanilamide itself, were showing an increased surface-activity as well, it would be interesting to ascertain, whether by introduction of a ring-system of strong surface activity (e.g., the naphthalene ring) compounds of therapeutic value could be obtained.

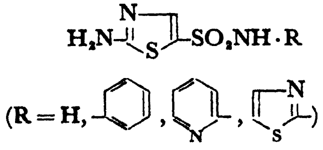
To that end Veldstra and coll. (A) examined naphthionamide, its pyridine- and its 4-methylpyrimidine-derivative:

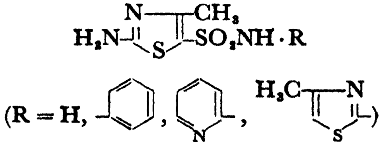


All these compounds however proved to be soluble with great difficulty, which hampered the testing of them. In vivo, against streptococci, none of the substances possessed an appreciable activity; in vitro this was markedly smaller than that of sulphanilamide, without mutual differences.

i. 2-Aminothiazole-5-sulphonamide and derivatives.

Backer and de Jonge (1943b, 1942) and also Backer and Keverling Buisman (1944) implicated in their researches a number of compounds, in which the benzene nucleus of the sulphanilamido-group is replaced by thiazole or 4-methyl-thiazole respectively; this in analogy with the comparable pyridine derivatives, already examined by Naegeli et al. (1938):

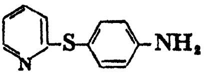




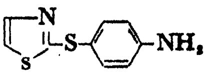
None of these compounds had any effect on pneumococci in vitro.

j. Sulphides.

Two heterocyclic derivatives of p-aminophenylthiol (Backer and Keverling Buisman, 1945):



,



did not develop any action against pneumococci.

k. Sulphones.

From the analoga of the strongly active 4,4′-diamino-diphenylsulphone, 4-aminophenyl-pyridyl-2′-sulphone proved to be twice as active as sulphanilamide, when tested on pneumococci in vitro, and the corresponding thiazolyl-derivative half as active. In vivo, however, neither of them were able to exercise any protecting action against pneumococcus-infections. 4-Aminophenyl-naphthyl-1′-sulphone and 4-aminophenyl-4′-aminonaphthyl-1′-sulphone were also prepared, but could not be tested in consequence of their slight solubility. (Backer and Keverling Buisman (1945).

l. Derivatives of p-aminobenzoic acid.

If the displacement theory of Woods and Fildes (sulphonamides displacing p-aminobenzoic acid, essential to bacterial metabolism) is correct, one might expect that other compounds than sulphonamides, chemically related to p.a.b.a., might display a bacteriostatic action.

Starting from this idea Backer and Sirks in an unpublished investigation tested a number of p-aminobenzoic acids, substituted in the nucleus, by adding them in the form of their sodium salts, to a 6 proc. ascites broth (without peptone) inoculated with pneumococci. By inspection of the turbidity of the cultures it was ascertained whether the bacteria did multiply or not.

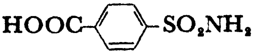
It was found that 2,4- and 3,4-diaminobenzoic acid showed a bacteriostatic action, which was counteracted by p.a.b.a.

Just like p.a.b.a., 2-chloro-4-amino- and 2-bromo-4-aminobenzoic acid are able to annihilate the bacteriostatic action of sulphanilamide.

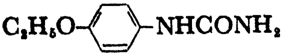
No activity whatever was displayed by 3-hydroxy-, 2-nitro-, 3-nitro-, 3-methyl- or 3,5-dimethyl-4-aminobenzoic acid.

m. Sulphonamide antogonists.

p-Sulphaminobenzoic acid ("parasaccharin"):



occurring as a contamination in the sweetening substance saccharin, and dulcin (p-ethoxyphenylurea):



both of which do not show any activitv against streptococci in vivo, nor in vitro, were tested especially for an eventual antagonistic action towards sulphonamides (VELDSTRA et al.,*A*).

For this purpose the substances, in varying quantities, were administered simultaneously with the protecting quantity of sulphapyridine or sulphamethylpyrimidine respectively, to mice infected with streptococci, after which it was ascertained whether the surviving time was shortened. Similar experiments were carried out with p-aminobenzoic acid and p-aminobenzoyl-l-glutamic acid, for the purpose of comparison. These experiments were also carried out in vitro.

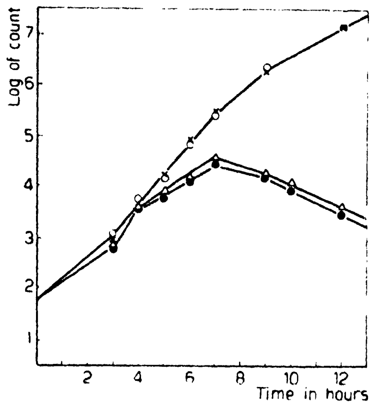


Fig. 30. Antagonistic action of p-aminobenzoic acid and of p-amino benzoyl-l-glutamic acid towards sulphonamides. (Growth-curves for S. pyogenus Ros.).

×——×	Control	
●——●	Sulphanilamide	60.10^{-5} mol/l
○——○	Sulphanilamide p-Aminobenzoic acid	60.10^{-5} mol/l 6.10^{-5} mol/l
△——△	Sulphanilamide p-Aminobenzoyl-l-glutamic acid	60.10^{-5} mol/l 12.10^{-5} mol/l

Neither p-sulphaminobenzoic acid nor dulcin showed any antagonistic activity in vivo or in vitro. The activity of p-aminobenzoic acid could be demonstrated clearly in both cases. p-Aminobenzoyl-l-glutamic acid proved to be nearly equivalent to p-aminobenzoic acid as an antagonist to sulphanilamides, when tested in vivo; in vitro, however, it showed practically no activity. (See Table V and Fig. 30). IVÁNOVICS (1944) with streptococci, and JULIUS and WINKLER (see page 31) with Bact. coli, streptococci and streptobacteria, also found p-aminobenzoyl-l-glutamic acid much less active than p-aminobenzoic acid in counteracting sulphanilamide. This result is quite contrary to that obtained by AUHAGEN (1943) with Streptobacterium plantarum, where p-aminobenzoyl-l-glutamic acid would be ten times as active as p-aminobenzoic acid. In the heading of this publication AUHAGEN indicates p-aminobenzoyl-l-glutamic acid as a more active sulphonamide-

TABLE V

ANTI-SULPHONAMIDE-ACTIVITY OF P-AMINOBENZOIC ACID AND OF P-AMINOBENZOYL-L-GLUTAMIC ACID, IN VIVO (MICE).

INFECTION WITH STREPT.PYOGENES ROSENBACH

Daily dose (in 10^{-5} moles)				Average surviving time after ten days (days)
sulpha-pyridine	sulpha-methyl-pyrimidine	p-amino-benzoic acid	p-amino-benzoyl-l-glutamic acid	
3		3		1
3		1,5		1
3		0,75		8,5
3		0,375		9,5
3				10
	3	3		1,5
	3	1,5		2
	3	0,75		8
	3	0,375		7·5
	3			10
	3		3	2
	3		1,5	3
	3		0,75	6
	3		0,375	5,5
Control (streptococcus infection only)				1

antagonist, from the text one would conclude, however, that the substance was tested as a growth-factor. It is desirable that these experiments should be repeated and the relationship between them definitely established.

From our results one might conclude that the derivative of l-glutamic acid does not react as such in vivo, but only the p-aminobenzoic acid, liberated by scission. This scission would not occur then in vitro. These interesting facts, important also to our outlook on the mechanism of the action of p-aminobenzoic acid and of the sulphonamides, will be studied further.

§ 2. ACTIVITY OF "SPOT TEST" REAGENTS

Assuming that the action of the sulphonamides does indeed consist in the displacing or inactivating of a factor, indispensable to the micro-organisms involved (i.c. p-aminobenzoic acid), it is conceivable that this object can be attained also by eliminating other factors, important to the micro-organism. With regard to heavy metals, which in certain cases are important in this respect, this might be achieved by using reagents, employed in micro analysis for the quantitative precipitation of these metals.

For this reason Veldstra et al. (A) examined rubeanic acid, dithizone, sodium diethyldithiocarbamate and dimethylaminobenzylidene rhodanine.

In vivo (against streptococci) no activity was observed.

Rubeanic acid proved to be toxic to mice in the concentration applied. The investigation will be extended to other pathogenic organisms.

In vitro, in the case of small inoculae, indications were obtained for a very small activity of sulphonamide-type (with a large lag-time), the reaction was the same practically for all compounds, disappearing however, if larger inoculae were used.

To arrive at a definite opinion these facts will have to be studied more in detail, the results of which will be published elsewhere (A).

§ 3. TABULAR SURVEY OF THE INVESTIGATED COMPOUNDS

Activity: For the comparison of the activities the following indications were used:

Vivo +++ = activity, equal to or greater than that of sulphapyridine.
++ = activity from $\frac{1}{2}$ — 1 of that of sulphapyridine
+ = ,, ,, $\frac{1}{4}$ — $\frac{1}{2}$,, ,, ,, ,,
± = ,, ,, 0 — $\frac{1}{4}$,, ,, ,, ,,
0 = inactive

Vitro ++ = activity, equal to that of sulphanilamide
+ = ,, from $\frac{1}{2}$ — 1 of that of sulphanilamide
± = ,, ,, 0 — $\frac{1}{2}$,, ,, ,, ,,
0 = inactive

P = Pneumococcus (Type I)
S = Streptococcus pyogenes Rosenbach

Literature references:

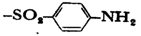
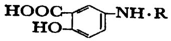
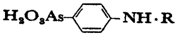
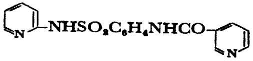
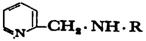
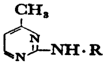
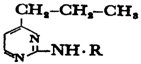
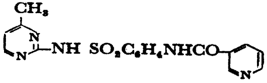
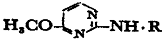
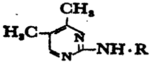
The figures in the last column but one refer to the Dutch publications, those in the last column to investigations, in which the compounds concerned were also studied.

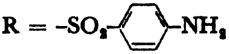
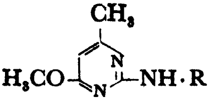
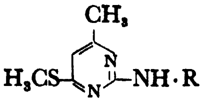
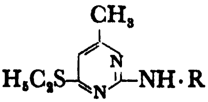
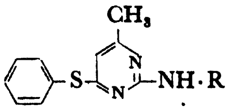
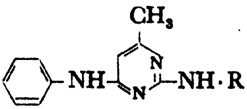
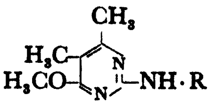
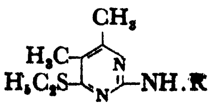
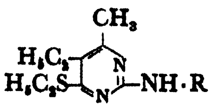
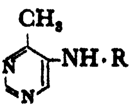
§ 4. BIOCHEMICAL INVESTIGATIONS

a. Action of sulphanilamide on enzymes.

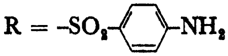
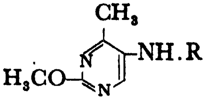
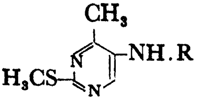
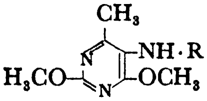
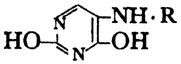
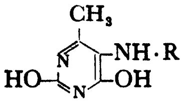
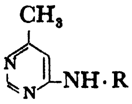
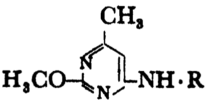
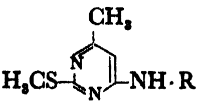
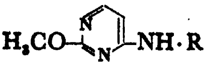
The communication of MANN and KEILIN (1940) that sulphanilamide even in a concentration of 2.10^{-6} mol/l inhibits to a large extent the carbonic anhydrase activity in blood solutions and seems to be rather specific in its action — other enzymes in the blood are not influenced in concentrations of sulphanilamide up to 2.10^{-2} mol/l — induced VAN GOOR (1942) to repeat these experiments with rabbit blood in vivo and in vitro. Whereas neither injection of 5 ml sulphanilamide 0.1 % into the maginale vein, nor intraperitoneal injection of twice the amount caused any enzyme inhibition, this occurred temporarily to an extent of 28 % when 50 ml of sulphanilamide 0.75 % were injected intraperitoneally. Assuming that the blood-volume of a rabbit is about 100 ml and that only 1/100 of the sulphanilamide is absorbed into the blood, a sulphanilamide concentration of

TABLE VI

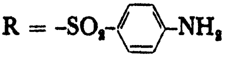
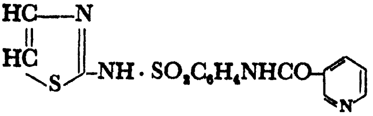
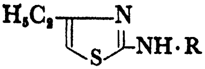
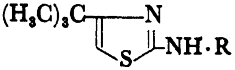
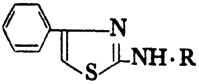
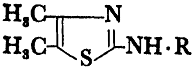
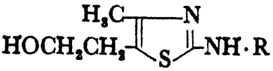
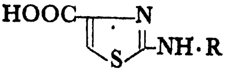
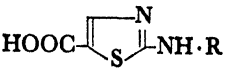
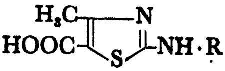
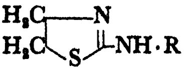
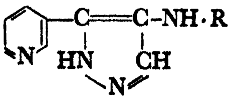
Nomenclature	Structure	m.p. °C	Activity				Ref.	
			vivo		vitro			
	R = 		P	S	P	S		
Sulphanilylcyanamide	$N{\equiv}C{-}NH\cdot R$	285		+		++	20, 21	56
Sulphanilylguanidine	$\begin{matrix}H_2N\\H_2N\end{matrix}\rangle C{=}N\cdot R$	188		±		++	21	47
Disulphanilylguanidine	$H_2N{-}C\langle\begin{matrix}NHR\\NR\end{matrix}$	221.^{5}–222.^{5}	o		++		9	
5-Sulphanilylaminosalicylic acid		211		o		++	21	30
4-Sulphanilylaminobenzene arsonic acid		220	o				1, 15	
N^4-nicotinylsulphapyridine		265	o	++		+	19	40
2-(Sulphanilylaminomethyl)-pyridine		130	o	o		+	19	41
2-Sulphanilylamino-4-methylpyrimidine		235–236	+++	+++		++	3, 12 19	37, 51 53
2-Sulphanilylamino-4-n-propylpyrimidine		216–217	o	+++		++	19	53
N^4-nicotinyl-2-sulphanilyl-amino-4-methylpyrimidine		248–250		++		+	21	
2-Sulphanilylamino-6-methoxypyrimidine		230.^{5}–231.^{5}	+				12	
2-Sulphanilylamino-4,5-dimethylpyrimidine		226.^{5}–227.^{5}	+++				12	28

Nomenclature	Structure 
2-Sulphanilylamino-4-methyl-6-methoxypyrimidine	
2-Sulphanilylamino-4-methyl-6-methylthiopyrimidine	
2-Sulphanilylamino-4-methyl-6-ethylthiopyrimidine	
2-Sulphanilylamino-4-methyl-6-phenylthiopyrimidine	
2-Sulphanilylamino-4-methyl-6-anilidopyrimidine	
2-Sulphanilylamino-4,5-dimethyl-6-methoxypyrimidine	
2-Sulphanilylamino-4,5-dimethyl-6-ethylthiopyrimidine	
2-Sulphanilylamino-4-methyl-5-ethyl-6-ethylthiopyrimidine	
5-Sulphanilylamino-4-methylpyrimidine	

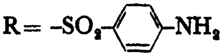
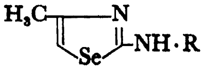
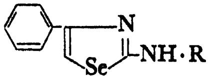
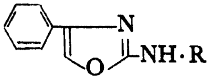
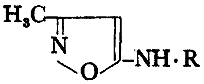
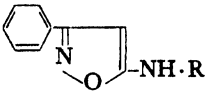
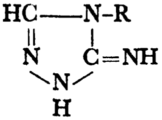
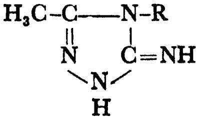
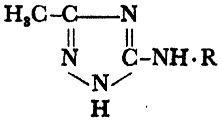
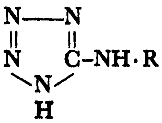
m.p. °C	Activity				Ref.
	vivo		vitro		
	P	S	P	S	
203.^{5}–204.^{5}	+ (toxic)				3, 12
197.^{5}–198.^{5}	++				3, 12
		++		+	21
139.^{5}–141	0				12
306–307	0				12
241–244	0				12
		+		±	21
		±		±	21
235.^{5}–236	+++				3, 12

Nomenclature	Structure 
5-Sulphanilylamino-2-methoxy-4-methylpyrimidine	
5-Sulphanilylamino-2-methylthio-4-methylpyrimidine	
5-Sulphanilylamino-2,6-dimethoxy-4-methylpyrimidine	
5-Sulphanilylamino-2,6-dioxypyrimidine	
5-Sulphanilylamino-2,6-dioxy-4-methylpyrimidine	
6-Sulphanilylamino-4-methylpyrimidine	
6-Sulphanilylamino-2-methoxy-4-methylpyrimidine	
6-Sulphanilylamino-2-methylthio-4-methylpyrimidine	
6-Sulphanilylamino-2-methoxypyrimidine	

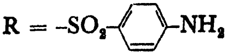
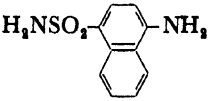
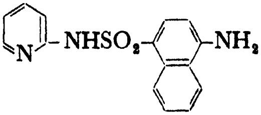
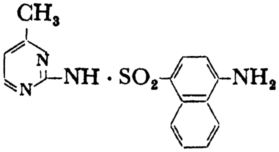
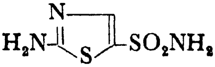
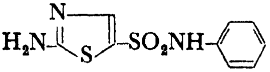
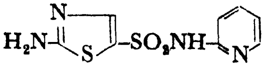
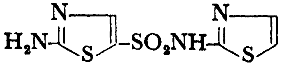
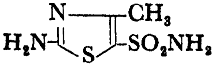
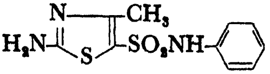
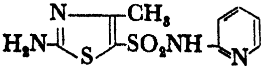
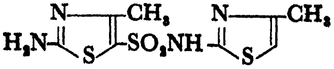
m.p. °C	Activity				Ref.	
	vivo		vitro			
	P	S	P	S		
191–192	very toxic				3, 12	
$240.^5$–241	0				12	
$195.^5$–196	toxic				12	
271	0				2, 12	51
283	±				2, 12	
209–211	+++				3, 12	
$188.^5$–$189.^5$	+++				3, 12	
213–$214.^5$	+++				12	
262–265	0				12	

Nomenclature	Structure 
N^4-nicotinyl-sulphathiazole	
2-Sulphanilylamino-4-ethylthiazole	
2-Sulphanilylamino-4-tert.butylthiazole	
2-Sulphanilylamino-4-phenylthiazole	
2-Sulphanilylamino-4,5-dimethylthiazole	
2-Sulphanilylamino-4-methyl-5-hydroxyethylthiazole	
2-Sulphanilylamino-4-carboxythiazole	
2-Sulphanilylamino-5-carboxythiazole	
2-Sulphanilylamino-4-methyl-5-carboxythiazole	
2-Sulphanilylamino-Δ^2-thiazoline	
4-Sulphanilylamino-5(3′-pyridyl-)pyrazole	

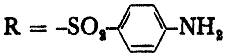
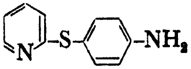
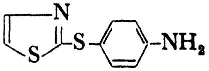
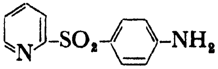
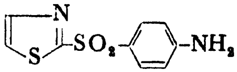
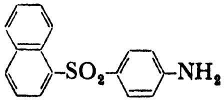
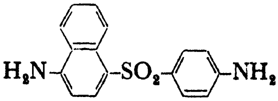
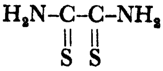
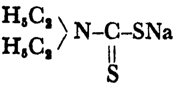
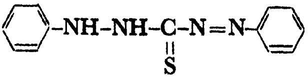
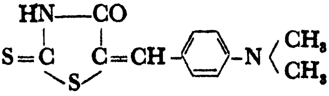
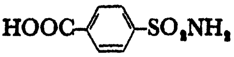
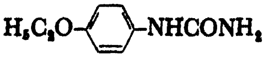
m.p.	Activity				Ref.	
	vivo		vitro			
	P	S	P	S		
279–280		++		++	21	29
152–153	+ (toxic)				1, 15	27
205–207	+ (toxic)				1, 15	
196–197	+ (toxic)				1, 15	
252–253	+				1, 15	33
$180.^{5}$–$181.^{5}$	0				4, 15	33
224–225	0				1, 15	
213–214	0				4, 15	
>150° ↗ CO_2	+				1, 15	33
209–210	++				1, 15	33, 50
210	0	0		++	19	

Nomenclature	Structure 
2-Sulphanilylamino-4-methylselenazole	
2-Sulphanilylamino-4-phenylselenazole	
2-Sulphanilylamino-5-phenyloxazole	
5-Sulphanilylamino-3-methylisoxazole	
5-Sulphanilylamino-3-phenylisoxazole	
N-sulphanilyltriazolone-imide-5	
N-sulphanilyl-3-methyl-triazolone-imide-5	
5-Sulphanilylamino-3-methyltriazole	
5-Sulphanilylaminotetrazole	

m.p. °C	Activity				Ref.	
	vivo		vitro			
	P	S	P	S		
236–237	+ (toxic)				1, 15	34
$209.^5$–$210.^5$	0				1, 15	
267–269	0				4, 15	36
164–165	±				4, 15	
173–174	++				4, 15	
186–187	0				5, 15	
209–210	0				5, 15	35
244–245	0				5, 15	
202–203	0	0		+	19, 20	compare 35, 38 51, 54 55

Nomenclature	Structure 
Naphthionamide	
2-Naphthionylaminopyridine	
2-Naphthionylamino-4-methyl-pyrimidine	
2-Aminothiazolesulphonamide-5	
(2-Aminothiazolesulphon-amino-5)benzene	
2-(2′-Aminothiazolesulphon-amino-5′)pyridine	
2-(2′-Aminothiazolesulphon-amino-5′)thiazole	
2-Amino-4-methylthiazole-sulphonamide-5	
(2-Amino-4-methylthiazole-sulphonamino-5)benzene	
2-(2′-amino-4′-methylthiazole-sulphonamino-5′)pyridine	
2-(2′Amino-4′-methylthiazole-sulphonamino-5′)-4-methyl-thiazole	

m.p. °C	Activity				Ref.	
	vivo		vitro			
	P	S	P	S		
209		0		+	21	31
216		0		+	21	
230		0		+	21	
160–161			0		7	
155.5–156.5			0		7	
225			0		7	
236			0		7	
175–176	0				6, 15	
135–136	very toxic				6, 15	
208–209	0				6, 15	
241	0				6, 15	

Nomenclature	Structure 
4-Aminophenyl-pyridyl-2′-sulphide	
4-Aminophenyl-thiazolyl-2′-sulphide	
4-Aminophenyl-pyridyl-2′-sulphone	
4-Aminophenyl-thiazolyl-2′-sulphone	
4-Aminophenyl-naphthyl-1′-sulphone	
4-Aminophenyl-4′-amino-naphthyl-1′-sulphone	
Rubeanic acid	 $H_2N-\underset{\underset{S}{\Vert}}{C}-\underset{\underset{S}{\Vert}}{C}-NH_2$
Sodiumdiethyldithiocarbamate	 $\left.\begin{matrix}H_5C_2\\H_5C_2\end{matrix}\right\rangle N-\underset{\underset{S}{\Vert}}{C}-SNa$
Dithizone	
Dimethylamino-benzylidene-rhodanine	
p-Sulphaminobenzoic acid	
p-Ethoxyphenylurea	

m.p. °C	Activity				Ref.
	vivo		vitro		
	P	S	P	S	
91			o		8
79			o		8
161	o		+++		8
153	o		+		8
203					8
265					8
		toxic		±	21
		o		±	21
		o		±	21
		o		±	21
283		o		o	21
173		o			21

2.10^{-4} mol/l inhibits only ¼ of the total activity. In vivo the inhibiting activity is therefore much smaller than that observed by Mann and Keilin in vitro.

Van Goor's in vitro experiments (0.1 ml sulphanilamide solution is added to 1 ml blood solution, diluted 1 to 100; 0.05 ml of this mixture is added then to 2 ml CO_2-bicarbonate mixture according to Brinkman, 1933) proved that a final sulphanilamide concentration of 5.10^{-5} mol/l produced an enzyme inhibition of 8 % only, at 2.10^{-3} mol/l this was not complete either (92 %). These effects too therefore are weaker than those obtained by Mann and Keilin.

The reversibility of the effect of sulphanilamide on red cells, already observed by Mann and Keilin, could be corroborated, as lysis of red cells, after they have been in contact with sulphanilamide solution and thereafter have been washed with saline, produces a blood solution of the same enzyme activity as that of a control, and as without washing with saline, the activity is reduced.

Finally van Goor proved that p.aminobenzoic acid is able to counteract the enzyme inhibiting action of sulphanilamide to a certain extent. This fact plays a part in the discussion on the mechanism of the action of the sulphanilamides and of p.aminobenzoic acid. (Compare Julius and Winkler, 1942).

Jonxis (1943) put the question, whether the symptoms of intoxication that sometimes appear in sulphonamide therapy might be caused by the above-mentioned influence of the sulphonamides upon the carbonic anhydrase.

In men this enzyme activity was reduced by 40 per cent when 1 gram of sulphanilamide was administered orally, but it returned to the normal level very soon. The effect of the drug is perceptible as soon as sulphanilamide can be traced in the blood, but before the sulphanilamide concentration has reached its maximum, however, it has disappeared already.

According to Jonxis the likeliest explanation for this is that the loose bond between the sulphanilamide and the enzyme is broken on further transformation of the drug.

As in the experiments in vitro of Mann and Keilin (1940) here too sulphapyridine has not any effect upon the anhydrase activity.

Supposing that the intoxication of the enzyme might demonstrate itself by influencing the transport of CO_2 — especially by a lowered delivery of CO_2 in the lungs — the total CO_2 content of the blood was determined. This did not, however, prove to be increased, but, on the contrary, it was lowered after administration of sulphanilamide.

The residual anhydrase activity is apparently sufficient to make the delivery of CO_2 function normally.

This result is in accordance with the observation of VAN GOOR (1942) that an enzyme inhibition of 28 per cent in rabbits exerts but a slight effect upon the respiration process.

In estimating the p_H of the blood it appeared, that one hour after administration of sulphanilamide (when the influence upon the carbonic anhydrase has already disappeared), the p_H shows a marked drop, namely to 6,8.

The same effect is shown by sulphapyridine and sulphathiazole. The cause of this phenomenon is not known. Here too JONXIS makes the "short living" intermediate products, formed in the further transformation of the sulphonamides, responsible for this.

§ 5. ANALYTICAL EXPERIMENTS

a. Quantitative determination of sulphonamides.

When VERHAGEN (1941 a, 1941 b) made his clinical and biochemical investigations on sulphapyridine, MARSHALL's (1937, 1938) method for the quantitative determination of sulphonamides — elaborated for sulphanilamide — was even then applied to three other substances only: sulphapyridine, sulphathiazole and sulphamethylthiazole. VERHAGEN demonstrated the suitability of this reaction in the case of sulphanilic acid, uliron and the local-anaesthetic novocain. The latter fact has to be taken into account, because of its being a possible source of error in sulphonamide-determinations, if, on punction, the anaesthetic should be mixed with the punctate. Afterwards the same method of determination was applied successfully to 2-sulpha-4-methylpyrimidine (Percoccide) (1942 a, 1942 b).

b. Diazo-reactions.

In connection with these investigations **Verhagen** (1941 a, 1941 b) tried if the sulphanilic acid used in Ehrlich's diazo-reaction (formation of a red azo-dye on addition of diazotized sulphanilic acid to alcalified urine) might be replaced by sulphanilamide or its derivatives. Urine samples of patients with a positive Ehrlich-test showed the same picture on application of sulphanilamide ,albucid, sulphapyridine, uliron, neo-uliron, sulphamethylthiazole and moreover with ephetonal or novocain when used instead of sulphanilic acid. In all these cases the coloration was visually the very same and equal to that obtained by means of sulphanilic acid. The dyes were not, however, tested spectroscopically. Apparently it is the hitherto unknown substance, which couples with the diazotized amine, that determines the shade of colour.

In the qualitative determination of bilirubin in body-liquors according to Hymans van den Bergh, where diazotized sulphanilic acid is also used (though in an acid medium), the eight compounds mentioned above proved to react in the same way as sulphanilic acid, without perceptible difference of colour. The only exception was in the case of sulphamethylthiazole, the diazonium salt of which, has an intensive colour of its own in an acid medium.

A more detailed study of these diazo reactions led to the observation that after administration of sulphapyridine a substance appears in the urine causing a positive Ehrlich reaction. The substance could not yet be identified; it was stated, however, that the reaction is not induced by sulphapyridine, acetylsulphapyridine, sulphanilamide, pyridine or 2-aminopyridine. It is very interesting that the relations described make it possible to obtain a positive diazo reaction in such a urine by the addition of sodium nitrite and hydrochloric acid only.

As the coloration obtained differs from that in the normal Ehrlich reaction, one may conclude that these reactions are not brought about by the same substance.

The possibility was entertained, to arrive at a quantitative colorimetric determination of this unknown substance by addition of an excess of diazonium salt. As the diazonium salts possess a colour of their own in an alcaline medium, a practically suitable method could not be elaborated.

c. Quantitative determination of p-aminobenzoic acid and of sulphonamides separately in mixtures.

In studying the mechanism of the action of sulphonamides, in which generally very much importance is attached to the interaction of these chemotherapeutics with p.aminobenzoic acid (see page 65), it seemed most desirable to dispose of a method for the quantitative determination of p.a.b.a. and sulphonamides separately in mixtures.

In search for this, HAVINGA (1941, 1944), made use of the experience acquired in the quantitative determination of novocain and other diazotizable anaesthetics, consisting in an extinctiometric determination of the dye, obtained by diazotizing and coupling with β-naphthol, after extraction with amyl alcohol. At an average p_H of 7, it proved to be possible to determine sulphapyridine, sulphamethylpyrimidine and also p.a.b.a.

To achieve a separation between p.a.b.a. and the sulphonamides it was considered utilizing the larger solubility in an alcaline medium of the azo-dye derived from p.a.b.a. Amyl alcohol was not suited as an extraction liquid; but when chloroform was used in its place it appeared that at a weakly alcaline reaction ($\sim$ 0.003 n NaOH) the coupling product of the sulphonamide went into the chloroform only, in which it may be determined in a colorimetric or extinctiometric way. After neutralization of the aqueous layer the coupling product of p.a.b.a. is extracted with amyl alcohol and determined in the very same way as in the case of novocain. The method is very suitable, also on micro-scale, as shown by the following table by HAVINGA (1944) (page 138).

Almost at the same time KIRCH and BERGEIM (1943) used a comparable method for the determination of p.a.b.a. in presence of sulphonamides, in which the diazotized solution is coupled with aneurin (thiamin). By extraction with isopropyl ether only the dye derived from p.a.b.a. is removed and that of the sulphonamide remains in the aqueous layer.

TABLE VII

DETERMINATION OF P.A.B.A. AND SULPHONAMIDES SEPARATELY IN MIXTURES

Mixture		Found	
p.a.b.a.	sulphapyridine	p.a.b.a.	sulphapyridine
68 γ	13·8 γ	65 γ	15·3 γ
6.8 γ	138 γ	6.8 γ	129 γ
68 γ	69 γ	61 γ	74 γ
2·74 γ	2·76 γ	2·46 γ	2·90 γ
p.a.b.a.	sulphamethylpyrimidine	p.a.b.a.	sulphamethylpyrimidine
68 γ	13·2 γ	71·5 γ	16.7 γ
6.8 γ	132 γ	8.9 γ	139 γ
27·4 γ	26.4 γ	26.9 γ	28.9 γ
137 γ	132 γ	136 γ	136 γ
p.a.b.a.	sulphanilamide	p.a.b.a.	sulphanilamide
137 γ	86 γ	121 γ	76 γ
6.8 γ	86 γ	7·4 γ	80 γ
68 γ	8.6 γ	67 γ	8.8 γ

LITERATURE

DUTCH LITERATURE: Cited on page

1. **Backer, H. J. and Jonge, J. de,** *De la préparation de quelques 2-sulfanilamidothiazoles et sélénazoles.* Rec. trav. chim., 60 (1941) 495 (a.) 110, 111
2. **Backer, H. J. and Grevenstuk, A. B.,** *Deux sulfanilamidopyrimidines.* Rec. trav. chim., 60 (1941) 502 (b) 107
3. **Backer, H. J. and Grevenstuk, A. B.,** *Quelques sulfanilamido-4-methylpyrimidines.* Rec. trav. chim., 61 (1942) 291 (a); *Quelques sulfanilamidopyrimidines substituées.* Rec. trav. chim., 64 (1945) 115 107
4. **Backer, H. J. and Jonge, J. de,** *Sulfanilamides renfermant les noyaux thiazole, oxazole et isoxazole.* Rec. trav. chim., 61 (1942) 463 (b) 110, 111

Cited on page

5. BACKER, H. J. AND JONGE, J. DE, *Dérivés de la sulfanilamide renfermant l'anneau 1,2,4-triazole.* Rec. trav. chim., 62 (1943) 158 (a) 111
6. BACKER, H. J. AND JONGE, J. DE, *2-Amino-4-méthylthiazole-sulfamide-5 et quelques dérivés.* Rec. trav. chim., 62 (1943) 163 (b) 114
7. BACKER, H. J. AND KEVERLING BUISMAN, J. A., *Quelques dérivés de l'acide 2-amino-thiazole-5-sulfamique.* Rec. trav. chim., 63 (1944) 228 114
8. BACKER, H. J. AND KEVERLING BUISMAN, J. A., *Préparation et activité sulfonamide de quelques amino sulfures et sulfones.* Rec. trav. chim., 64 (1945) 102 . . . . 114, 115
9. BACKER, H. J. AND MOED, H. D., unpublished, (A) 105
10. BRINKMAN, R., *The occurrence of carbonic anhydrase in lower marine animals.* J. Physiol., 80 (1933) 171 134
11. GOOR, H. VAN, *The inhibition of carbonic anhydrase by sulphanilamide.* Onderzoekingen Physiol. Lab. Rijksuniversiteit Utrecht (8) II, (1942) 142. Enzymologia XI (1944) 174 . . . . 119, 135
12. GREVENSTUK, A. B., *Synthese en chemotherapeutisch onderzoek van sulfanilamidopyrimidinen.* Thesis Groningen 1942 107
13. HAVINGA, E., *Über die extinktiometrische Bestimmung des Novocains und anderer diazotierbarer Anaesthetika.* Rec. trav. chim., 60 (1941) 897 137
14 HAVINGA, E., *Die Bestimmung der p.Aminobenzoesäure und des Sulfapyridins, bzw. ähnlicher Verbindungen nebeneinander.* Rec. trav. chim., 63 (1944) 243 137
15. JONGE, J. DE, *Heterocyclische derivaten van sulfanilamide en hun chemotherapeutische activiteit.* Thesis Groningen 1942 . . . 110, 114
16. JONXIS, J. H. P., *De vergiftiging der koolzuuranhydrase door sulfanilamide-verbindingen.* Ned. Tijdschr. v. Geneesk., 87 (1943) 1669 134
17. JULIUS, H. W. *Een methode voor het tellen van levende bacteriën, vervanging voor de plaatmethode.* Ant. v. Leeuwenhoek, 5 (1938) 28 105
18. JULIUS, H. W. AND WINKLER, K. C., *On the action of sulfanilamide VIII. The mechanism of its action.* Ant. v. Leeuwenhoek, 8 (1942) 139 134
19. VELDSTRA, H. AND WIARDI, P. W., *Synthesis and activity of some heterocyclic sulphanilamide derivatives I.* Rec. trav. chim., 61 (1942) 627 . . . . 106, 109, 110, 111, 112
20. VELDSTRA, H. AND WIARDI, P. W., *Researches on sulphanilamide derivatives II. On 5-sulphanilylamino-tetrazole.* Rec. trav. chim., 62 (1943) 661 112
21. VELDSTRA, H. and coll., *Researches on sulphanilamide derivatives VII. The in vivo and in vitro activity of a series of sulphanilamide derivs. and other compounds on Streptococcus pyogenus Rosenbach.* To be published in Rec. trav. chim. (A) 105, 106, 109, 110, 113, 116, 118

Cited on page

22. Verhagen, H., *Het sulfapyridine. Klinische en biochemische onderzoekingen.* Thesis Amsterdam 1941 (a) 135, 136
23. Verhagen, H., *Diazo-reacties.* Ned. Tijdschr. v. Geneesk., 85 (1941) 1562 (b) 135, 136
24. Verhagen. H., *Onderzoekingen over sulfamethylthiazol en sulfamethylpyrimidine.* Ned. Tijdschr. v. Geneesk., 86 (1942) 1171 (a) 135
25. Verhagen, H., *Pharmacologische onderzoekingen over het sulfamethylthiazol (Ultraseptyl) en sulfamethylpyrimidine (Percoccide) vergeleken met het sulfapyridine (Dagenan) benevens enkele klinische ervaringen.* Ned. Tijdschr. v. Geneesk., 86 (1942) 3048 (b) . . 135

Literature from other countries:

26. Auhagen, E., *p-Aminobenzoylglutaminsäure, ein gegen Sulfonamide wirksameres Derivat des Vitamins H'.* H. S. Z. physiol. Chem., 277 (1943) 197 117
27. Bergheim, H., Coy, N. H. and Lott, W. A., *2-(p.Aminobenzenesulfonamino-) 4-ethyl-thiazole.* J. Amer. Chem. Soc., 62 (1940) 1873; C. (1940) II, 2605 127
28. Caldwell, W. T., Kornfeld, E. C. and Donnell, C. K., *Substituted 2-sulphanilylaminopyrimidines.* J. Amer. Chem. Soc., 63 (1941) 2188; C. (1942) I, 485 121
29. Cooper, F. B., Gross, P. and Lewis, M., *Chemotherapeutic evaluation of some N^1- and N^4-heterocyclic derivatives of sulphanilamide.* Proc. Soc. exp. Biol. Med., 47 (1941) 508; C. (1942) I, 3015 127
30. Crossley, M. L., Northey, E. H. and Hultquist, M. E., *Sulphanilamide derivatives I. Aminoarylsulphonamidoarylsulfonic acids and aminoarylsulfonamidoaryl carboxylic acids.* J. Amer. Chem. Soc., 60 (1938) 2217 121
31. Goldyrev, L. N. and Postovskii, J. Ya., *The structure and the pharmacological action of substances containing the sulfonamide group I.*
    The preparation of sulfonamides of anilin, a-naphthylamin and o-, m- and p-toluidines. The preparation of some azo-dyescontaining the sulfonamide group. J. applied Chem. (U.S.S.R.), 11 (1938) 316; Chem. Abstr., 32 (1938) 5800 131
32. Ivánovics, G., *Experimentelle Chemotherapie der bakteriellen Infektionen.* Acta Medica Szeged, Tom. XII, fasc. 1 (1944) . . . 117
33. Jensen, K. A. and Thorsteinsson, Th., *Sulfanilylderivater af heterocykliske aminer II Tiazol- og Tiazolinderivater.* Dansk Tidsskr. Farm. 15 (1941) 41 127
34. Jensen, K. A., *Sulfanilylderivater af heterocykliske aminer IV. Derivater af selenazole.* Dansk Tidsskr. Farm., 15 (1941) 197 (a) 129

Cited on page

35. Jensen, K. A., *Sulfanilylderivater af heterocykliske aminer V. Derivater af 1,2-diazol (pyrazol), 1,2,4-triazol og tetrazol.* Dansk Tidsskr. Farm., 15 (1941) 299 (b.) 111, 129
36. Jensen, K. A., *Sulfanilylderivater af heterocykliske aminer VI. Oxazol- og oxazolinderivater.* Dansk Tidsskr. Farm., 16 (1942) 1 129
37. Jensen, K. A., and Falkenberg, P., *Sulfanilylderivater af heterocykliske aminer VIII. Pyrimidinderivater.* Dansk Tidsskr. Farm., 16 (1942) 141 121
38. Jensen, K. A. and Rosenlund Hansen, O., *Ueber 5-Sulfanilamidotetrazol.* Rec. trav. chim., 62 (1943) 658 . . . . . 112, 129
39. Kirch, E. R. and Bergeim, O., *Determination of p.aminobenzoic acid.* J. Biol. Chem., 148 (1943) 445; C. (1944) I, 40 137
40. Kolloff, H. G. and Hunter, J. H., *Sulphanilamide compounds III. N^1-Hetero-oylderivatives of N^4-substituted sulphanilamides.* J. Amer. Chem. Soc., 62 (1940) 1646; C. (1940) II, 2603 . . 121
41. Kolloff, H. G. and Hunter, J. H., *Sulphanilamidederivatives VI. N^4-Acyl-N^1hetero-oyl sulphanilamides and N^1-hetero-oyl sulphanilamides.* J. Amer. Chem. Soc., 63 (1941) 490; C. (1941) II, 2323 121
42. Läuger, P. and Martin, H., *Ueber Irgafen. Ein Beitrag zur Konstitutionsspezifität.* Schweiz. med. Wochenschr., 73 (1943) 399 108
43. Mann, T., and Keilin, D., *Sulphanilamide as a specific inhibitor of carbonic anhydrase.* Nature, 146 (1940) 164 . . . . . . 119, 134
44. Marshall, E. K., *Determination of sulfanilamide in blood and urine.* Proc. Soc. exp. Biol. Med., 36 (1937) 422; J. Biol. Chem., 122 (1937) 263 135
45. Marshall, E. K., Emerson, K. and Cutting, W. C., *Para-aminobenzene sulfonamide. Absorption and excretion. A method of determination in urine and blood.* J. Amer. Med. Assoc., 108 (1937) 953 135
46. Marshall, E. K. and Litchfield, J. T., *The determination of sulfanilamide.* Science, 88 (1938) 85 135
47. Marshall, E. K., Bratton, A. C., White, H. J. and Litchfield, J. T., *Sulphanilylguanidine. Chemotherapeutic agent for intestinal infections.* Bull. John Hopkins Hosp., 67 (1940) 163; C. (1941) I, 1835 . . . . 105, 121
48. Marshall, E. K., Bratton, A. C., Edwards, L. B. and Walker, E., *Sulfanilylguanidine in the treatment of acute bacillary dysentery in children.* Bull. John Hopkins Hosp., 68 (1941) 94 . . 105
49. Naegeli, C., Kündig, W. and Brandenburger, H., *2-Aminopyridin-5-sulfonsäureamid und einige Abkömmlinge.* Helv. Chim. Acta, 21 (1938) 1746 114
50. Raiziss, G. W. and Clemence, Le Roy W., *2-Sulphanilylaminothiazoline.* J. Amer. Chem. Soc., 63 (1941) 3124; C. (1942) II 404 127
51. Roblin, R. O., Williams, J. H., Winnek, Ph. S. and English, J. P., *Chemotherapy II. Some heterocyclic sulphanilamide derivatives.*

Cited on page

J. Amer. Chem. Soc., 62 (1940) 2002; C. (1940) II, 3476 . . . 108, 111, 121, 125, 129

52. Rossi, E., *Das Sulfaguanidin (S.G.) in der Behandlung intestinaler Affektionen beim Kinde.* Schweiz. med. Wochenschr., 74 (1944) 279 105

53. Sprague, J. M., Kissinger, L. W. and Lincoln, R. M., *Sulphanilamide-derivatives of pyrimidines.* J. Amer. Chem. Soc., 63 (1941) 3028; C. (1942) II, 529 121

54. Tappi, G., *Ueber einige heterocyclische Derivate Sulfanilamids.* Rec. trav. chim., 62 (1943) 207 . . . . 112, 129

55. Tappi, G. and Migliardi, C., *Ricerche su alcuni derivati eterociclici della solfanilamide.* Arch. Sci. biol. ital., 27 (1941) 170; C. (1941) II, 2935 . . . . 112, 129

56. Winnek, Ph. S., Anderson, G. W., Marson, H. W., Faith, H. E. and Roblin, jr, R. C., *Studies on chemotherapy V. Sulphanilylcyanamide and related compounds.* J. Amer. Chem. Soc., 64 (1942) 1682; C. (1943) II, 1794 121

IV. PHARMACOLOGICAL, IMMUNOLOGICAL AND CLINICAL INVESTIGATIONS

§ I. PHARMACOLOGICAL INVESTIGATIONS

a. Distribution, Excretion.

MULDER, VAN DER ZOO DE JONG and SNYMAN (1940) reported an extensive study on resorption and excretion of sulphapyridine, estimating the substance and its acetyl derivative in blood, urine and spinal fluid by the method of the second author (VAN DER ZOO DE JONG, 1939) (a modified diazo-coupling procedure).

In 12 patients after a dose of 2–4 g sulphapyridine excretion was completed in five to seven days; 65.3–87.9 % of the total amount was excreted in the urine.

Blood concentration is dependent on resorption velocity, diffusion from the blood in the tissues, renal excretion and acetylation (the acetylated product being inactive). Maximal concentration after a single dose of 2 g, was reached in 4–6 hours and amounted to 2.5–3.75 mg %. Large initial doses kept the blood concentration on a higher level than several smaller doses. With daily oral doses, 6 g produced a sufficient concentration of 2.6–13.8 mg % in the blood, while 3 g daily kept the blood concentration on 1.1–2.2 mg % only.

There is a large individual difference in velocity of acetylation. The concentration in the spinal fluid is 40–60 % of the concentration in the blood.

Another extensive study in this field by VERHAGEN (1941, 1942) was already discussed in Chapter III.

PINKHOF (1939) estimated sulphapyridine in tears, humour (aqueous and vitreous), 2½ hours after a single oral dose. The blood concentration at that time was 3 mg, whereas in the studied fluids the concentration amounted to about 0.75 mg %.

Rectal administration of sulphanilamides was suggested on clinical grounds by TILANUS (1942).

b. Cyanosis.

KRAAN and Miss VAN VEEN (1942) estimated methemoglobin with the hemoxymeter in 37 patients during sulphapyridine or sulphathiazole treatment. In 34 cases methemoglobin was found. This substance indeed is the main cause of cyanosis. In the three remaining cases and in five of the 34 already mentioned a serious cyanosis existed, independent of the amount of methemoglobin. The authors suppose the verdohemochromogen of HEUBNER (sulphhemoglobin) and other degradation products to be the cause of these intoxications. After treatment with sulphanilamide more serious intoxications with a higher methemoglobin content of the blood were observed than with sulphapyridine, which again seemed more toxic in this respect than sulphathiazole. JONXIS (1943), after estimating methemoglobin with his own method (JONXIS, 1942) claims that cyanosis cannot be due to methemoglobin, which often disappears from the blood when sulphapyridine is still present.

c. Antipyretic action.

KNOPPERS (1942) studied the antipyretic action of various sulphanilamides in rats. This action is due to a reduction of heat production, probably by interference in cell metabolism, and varies for various derivatives independent of their bacteriostatic properties. The antipyretic action of sulphathiazole is not counteracted by p.a.b.a., from which the author concludes that the point of attack is different principally from that of the bacteriostatic action.

d. Action on carbonic anhydrase.

JONXIS (1943, 1944) reports the inhibition of carbonic anhydrase in patients to be slight after sulphanilamide treatment and absent after sulphapyridine treatment. The blood-alcali-reserves and p_H decrease for a short time after sulphanilamide treatment (comp. also Chapter III, page 134).

Though of no clinical interest, the studies of BAKKER (1942) are important in so far as the observed turbidity of lenses in the surviving rabbit eye under the influence of sulphanilamide, shows the importance of carbonic anhydrase for the metabolism of this organ.

e. Tissue metabolism.

No influence of sulphanilamide on oxidation in vitro of brain, kidney and liver suspensions of normal guinea-pigs was observed by PUNT (1943).

§ 2. IMMUNOLOGY

ZELDENRUST and VERLINDE (1942) report that sulphapyridine is not an anaphylactogen for rabbits and that the antigenic properties of lung protein are not altered by sulphapyridine.

The lung-extracts of rabbits infected with pneumonia (pasteurella), and treated with sulphapyridine are, however, more strongly anaphylactogenic than the lung-extracts of untreated controls, so the authors sustain the view that local necroses in lungs of patients with lobar pneumonia, treated with sulphapyridine, are due to local anaphylactic phenomena, whilst a general anaphylactic reaction should be the cause of unexpected death in these patients.

§ 3. CLINICO-THERAPEUTICAL RESULTS

a. Streptococci.

In an early period of the new chemotherapy Miss VISSER (1939), in a statistical analysis, reported good results with sulphanilamide treatment in 200 patients with erysipelas as compared with 200 untreated cases. Lethality decreased from 6 to 2 %. The local redness vanished in four days in treated cases, in nine days in the control group (probable average).

KRÖGER-WIBAUT and HERDERSCHÉE (1940) reported the ineffectiveness of sulphanilamide in 500 cases of scarlatina.

VAN VEEN (1945) obtained good results with sulphathiazole treatment of purulent skin infections.

HULST (1944) cured two cases of endocarditis lenta with prontosil and sulphatiazole respectively.

b. Pneumococci.

104 cases of ulcus serpens were successfully treated with sulphapyridine by Keiner (1942, 1943).

Miss van Ormondt (1940) treated 49 cases of pneumonia in children with sulphapyridine, whereas Munk (1940, 1941) reported good results in treating 50 cases of pneumonia (and bronchopneumonia) in infants with sulphanilamide.

Pneumococci-meningitis was treated with sulphapyridine by Schoonhoven van Beurden and Loonstra (1940) (4 cases) and Weersma (1940) (2 cases). Gerlings and Kröger-Wibaut (1940) (cit. Gerlings, 1940) report a case of pneumococci-meningitis which was cured by the combination of sulphapyridine and type-specific antiserum only.

A case of sepsis was cured with sulphapyridine by van Alphen de Veer (1940). See also Mulder (1940, 1941).

c. Meningococci.

22 cases of meningitis due to meningococci were treated with sulphapyridine by Schoonhoven van Beurden and Loonstra (1940). Case mortality was reduced from 90 % to 31 %, compared with 17 previous cases. Still better results were reported by Weersma (1940) (10 cases) and by Kuenen and Haex (1940) (5 cases). Haex and Korthof (1940) report by morphological and cultural methods the disappearance of meningococci from the spinal fluid within three hours after intramuscular injection of soluble sulphapyridine.

Sepsis due to meningococci (spotted fever) was cured with sulphanilamide by Burkens (1940) and by Kramer (1941).

d. Gonococci.

The beneficial results of sulphapyridine treatment of acute gonorrhoea was reported by Stigter (1941) (77 cases) and by Westerhof (1941) (96 cases) whereas Polano and Neering (1942) reported good results with sulphathiazole treatment in the same disease (165 cases). Those long series of cases of gonorrhoea are a peculiarity of war time. Formerly primary infections of gonorrhoea were rather exceptional in our country.

Polano (1944) treated cases of gonorrhoea, resistant to sulphathiazole (20 g in four days), with high doses of sulphapyrimidine (Pyrimal, 30–40 g in three days) and was able to cure 3 out of 12 patients after one treatment and 5 after two treatments. Still better were the results with sulphamethylpyrimidine (Percoccide, 45 g in four days) with the aid of which, in 23 similar cases 11 were cured after one, 4 after two and 3 after three treatments (Polano, 1945).

De Boer (1942), Prakken and Stigter (1942), Beek (1944) and Prakken, Verwoerd and Carol (1944) report on the combined fever- and chemotherapy of resistant cases of gonorrhoea.

e. Other micro-organisms.

Further reports deal with the treatment of sympathetic ophthalmia (Colenbrander, 1939), pneumonia by klebsiellae (Loopuyt and Lubsen, 1943), infections with hemophilus influenzae (Mulder, 1941; Weyers, 1939; Halbertsma, 1939), gas gangrene (Wielenga, 1939). Good results were also reported in erythema exsudativum multiforme (van der Velde, 1940). In morbus Dühring with sulphanilamide, transient cures only could be effected (Vleugels Schutter, 1941). Most remarkable is a report by Royer (1941) who cured an agranulocytosis, following a streptococcic infection, with sulphanilamide! The influence of sulphanilamide on several otogenic infections was discussed by Stigter (1941). Van Lookeren Campagne and Westenberg (1944) in treating diphtheria with sulphathiazole concluded that the course of the disease was not influenced.

The treatment of dysenteric infections of the bacillary type is amply discussed by Lopes Cardozo (1940). His observations refer chiefly to results with sulphapyridine. It has to be kept in mind, that sulphanilylguanidine was not yet available. Nevertheless his results were more than encouraging. Dysentery in the Netherlands rarely presents itself as so murderous a disease as it can be elsewhere. Its economical significance under certain circumstances can be considerable, however. The chief gain lies in a substantial reduction of the duration of the disease, and of its wearing character. A good deal of attention was given to the in vitro action of the sulphonamides on the dysentery bacillus. The substance was added in different concentrations to agar plates. The limit of growth inhibition was estimated and the influence of the temperature was studied.

A theory on sulphonamide action and the action of chemotherapeutics in general has been elaborated in his discussions on the results. The author introduced the term "devitalization" (bacterio-degeneration) as a description of the characteristic aspect of the action of the chemo-therapeutics under discussion. The merits of this view will be discussed elsewhere.

Lopes Cardozo (1944) obtained very good results with sulphathiazole in the treatment of bacillary dysentery in children.

The same author (1945), on account of several arguments, doubted if the "specific intestinal" sulphonamides, such as sulphanilylguanidine and succinyl-sulphathiazole, are more active in intestinal infections than e.g., sulphapyridine and sulphathiazole.

f. Toxic reactions.

Hematuria due to concretions of acetylsulphapyridine, and anuria as serious complications of sulphapyridine treatment was reported by Boerema (1941) who pleaded for cystoscopy, Enneking and Prick (1944), Hulst and Cornelis (1940), Snapper, Liu and Chung (1939), Sutorius (1940, 1941). Sodium bicarbonate injections — 500 ml of a 4 % solution in water intravenously — were suggested as a therapy by Enneking and Prick (1944).

Ischiadicus-neuritis and polyneuritides after sulphapyridine- (Diepen, 1942) and sulphamethylthiazole treatment (Droogleever Fortuyn and Hoorweg, 1943; Enneking and Prick, 1944) were reported. The latter authors insist on the toxic character and the bad prognosis in this kind of neuritis.

Binkhorst (1945) observed a case of neuro-retinal degeneration, causing blindness, after administration of sulphamethylthiazole. Compare also Klessens (1941).

LINDEBOOM (1941) describes four cases of agranulocytosis after sulphapyridine. Another case was reported by SAMSON (1940).

For cyanosis see under pharmacological investigations.

g. New compounds.

Of late the new synthesized compound sulphamethylpyrimidine (Percoccide, comp. p. 109) was extensively tested in several Dutch clinics. Most of the work is not yet published but has been kindly put at our disposal, ready for print, by the authors. The general impression is that this compound is a real advancement in the sulphonamide therapy. Its therapeutic power is at least equivalent to that of sulphapyridine and sulphathiazole. It acts just as these substances, on streptococci, pneumococci and gonococci. Its effect on urinary infections is of the same value, even when caused by the colon bacillus. It is recorded that in some cases where the pyridine and thiazole compounds seemed to have failed, a final result could be obtained with the methylpyrimidine derivative. The most striking gain, however, lies in its relatively low toxicity and in its favourable properties as to the point of blood concentration and excretion in the urine. After one dose of 3 g (VAN DER MEER, 1944) the blood level is raised to 7 mg % in 6 hours (0.6 mg % being acetylated), whereas sulphapyridine and sulphathiazole under the same conditions give values of 3.4 and 3.0 mg % (1.2 and 0.5 mg % being acetylated respectively). After 24 hours the blood level of sulphamethylpyrimidine still is 1.7 mg % (0.2 mg % acetylated) against 0.7 mg % (0.3 mg % acetylated) in the case of sulphapyridine, and none in the case of sulphathiazole. The values are the average of determinations in 12, 6 ill and 6 normal persons, respectively. (Compare VAN DER MEER (1945), which paper appeared just before finishing printing of this review).

JOSSO (1945) determined the excretion in the urine and found the excretion of sulphamethylpyrimidine much slower than that of sulphapyridine. The tendency to nausea was less pronounced than in sulphapyridine. Urine concretions and morphological blood-alterations were minute, and it appeared that with equal doses the concentration of sulphamethylpyrimidine in the blood and probably also in the liquor is higher than that of sulphapyridine. It must be stated, however, that the number of patients treated is too small to allow of a definite judgment.

The results with sulphanilylguanidine (Dutch preparation: Sulfentidine) are, as can be expected from foreign records, encouraging but not yet extensive enough for detailed reportage. On account of the results in 63 cases of bacillary dysentery, 33 of which were treated with sulphanilylguanidine without a distinct effect, VAN DER WEY (1944) objected to routine treatment of this disease with sulphonamides. (Compare LOPES CARDOZO (1945), page 148).

WALDECK (1945) obtained very good results with the new sulphonamide euvernil (sulphanilylcarbamide) in thirteen urological and surgical patients, where in certain cases other drugs, such as prontosil rubrum, ultraseptyl and cibazol, failed. (Compare LOPES CARDOZO (1945, a), who criticised this statement.)

h. Local applications of sulphonamides.

The question of local applications of the sulphonamides has been amply discussed by QUARLES VAN UFFORD (1945). His researches were undertaken with the special aim of studying the influence of sulphonamides on wound healing. The uninfected, i.e., the uncomplicated healing of standardized skinwounds in rats is little retarded by sulphanilamide. Sulphapyridine and sulphathiazole have a more retarding effect on the process of healing than sulphanilamide. Other substances (in unguents, solutions, powders, etc.) in general have no influence or cause a more or less marked delay. The addition of any considerable amount of boric acid is definitely unfavourable. Application of short duration allows the wound to recover almost completely. Experimental wound infection could be realised only by a strain of B. proteus. The author failed to realise a sure and reproducible infection with streptococci or staphylococci. The proteus infection could be favourably influenced when an intensive contact with the drug was assured by taking away the scab every time. If not, beneficial effect was practically absent. The preventative application with the aim of combating an infection before it had settled in the wound was effective as compared with its control. His conclusions are in agreement with the general clinical experiences, that the sulphonamides are a real gain when applied early to wounds, i.e., before the occurrence of wound infection. The treatment should be of no longer duration than may be considered as indispensable for suppressing the initial infection. Then the process of healing should be left to its self. A favourable influence after that can be by no means considered as sure. Substances beneficial to the natural process of wound healing are as yet unknown. In any case they cannot be found under the sulphonamides.

Rijnders (1945) obtained favourable results with the intraperitoneal application of sulphamethylthiazole (Ultraseptyl) and sulphamethylpyrimidine (Percoccide) respectively in quantities of 12–24 grams — to combate or to avoid peritonitis. By the local application of these sulphonamides in colon and rectal surgery it was possible to simplify some of the usual surgical methods. The number of the treated cases is not very extensive yet, but the results obtained are very encouraging.

i. Comparative clinical investigation of a number of sulphonamides.

An interesting series of experiments was made by Verhagen (A) when he compared the clinical activity of a number of sulphonamide compounds of different type, and with a real or alleged reputation. Parallel with estimations of the therapeutic activity on cystitis caused by the colon bacillus, this being rather uniform and certain to combate, Verhagen determined the concentration in the urine of the different drugs. He found a perfect parallelism between the therapeutic effect and the concentration of the diazotizable substance. The series is an affirmation of the general view that the amino group in paraposition to the sulphonamide-grouping is essential. If it is not present in the original molecule, the action is limited to that part of the dose, that is altered by the body metabolism to liberate the amino grouping. Thus the experiments give an excellent direct proof on a clinical base.

Dutch literature has been enriched by an almost complete monograph from the hand of Lindeboom (1940, reprinted 1944). He rendered a valuable service to Dutch practitioners by this review of the most important literature on the subject, thus giving the clinicians a useful guide for their daily work.

LITERATURE

Cited on page

Alphen de Veer, M. R. van, *Pneumococcussepsis, genezen na toediening van hooge doses dagénan.* Ned. Tijdschr. v. Geneesk., 84 (1940) 5045 146

Bakker, A., *Lenstroebelingen veroorzaakt door sulfanilamide (prontosil album).* Ned. Tijdschr. v. Geneesk., 86 (1942) 1379 144

Balen, G. F. van, and Vliet, D. van der, *Agranulocytose met longaandoening.* Geneesk. Gids, 18 (1940) 326 . . . .

Beek, C. H., *Over het mislukken der chemotherapie bij gonorrhoe.* Ned. Tijdschr. v. Geneesk., 86 (1942) 303 147

Beek, C. H., *Over de behandeling van tegen chemotherapie resistente gonorrhoe.* Ned. Tijdschr. v. Geneesk., 88 (1944) 57 147

Berge, B. S. ten, *Puerperaal-infectie en de bestrijding met sulfonamiden.* Geneesk. Gids, 21 (1943) 31, 45 . . . .

Binkhorst, C. D., *Blindheid na toediening van ultraseptyl, 2-(p-aminobenzolsulfamido-4)-methylthiazol.* Ned. Tijdschr. v. Geneesk. Noodnummer XXXII (1945) 418 148

Boer, S. K. de, *Over de behandeling van tegen chemische therapie resistente gonorrhoe.* Ned. Tijdschr. v. Geneesk., 86 (1942) 2517 147

Boerema, I., *Anurie door dagénan.* Ned. Tijdschr. v. Geneesk., 85 (1941) 1831 148

Brouwer, B., *Clinische en pathologisch-anatomische demonstraties betreffende organische zenuwziekten. Demonstratie van een patiënt met een hersenabces, voorloopig behandeld met een injectie van soludagénan.* Ned. Tijdschr. v. Geneesk., 84 (1940) 3623 . . . .

Burkens, J. C. J., *Genezen meningococcussepsis, waarbij groote doses sulfanilamide zijn toegepast.* Ned. Tijdschr. v. Geneesk., 84 (1940) 1639 146

Bijlmer, L. and Mulder, J., *Resistentie van den sulfapyridinevasten pneumococcusstam Amerika type I tegen sulfathiazol, sulfamethylthiazol en sulfamethylpyrimidine.* Ned. Tijdschr. v. Geneesk., 86 (1942) 2626 . . . .

Cited on page

Colenbrander, M. C., *Belangrijke verbetering van sympathische ophthalmie door gebruik van dagénan en inspuitingen van leverpraeparaten.* Ned. Tijdschr. v. Geneesk., 83 (1939) 5939 147

Diepen, R. B. W. F. M., *Neuritis ischiadica na intramusculaire toediening van sulfapyridine (dagénan).* Ned. Tijdschr. v. Geneesk., 86 (1942) 1415 148

Droogleever Fortuyn, J. and Hoorweg, P. G., *Polyneuritis na gebruik van sulfametylthiazol (ultraseptyl).* Ned. Tijdschr. v. Geneesk. 87 (1943) 742 148

Enneking, J. A. M. J. and Prick, J. J. G., *Onaangename verwikkelingen bij de behandeling met sulfanilamide-verbindingen (ultraseptyl e.a.).* Geneesk. Bladen 40e Reeks, VIII, (1944) 241 148

Gerlings, P. G. *Dagénanbehandeling bij keel-, neus- en oorziekten.* Ned. Tijdschr. v. Geneesk., 84 (1940) 1616 146

Gerlings, P. G., *De resultaten bij dagénanbehandeling.* Ned. Tijdschr. v. Geneesk., 84 (1940) 1860 146

Gerlings, P. G., *Chemotherapie bij otitis media.* Ned. Tijdschr. v. Geneesk., 85 (1941) 2083 146

Gerlings, P. G. and Kröger-Wibaut, C. M., *Een geval van pneumococcus-meningitis na acute otitis media, genezen door operatie van Ramadier, dagénan en specifiek anti-pneumococciserum.* Ned. Tijdschr. v. Geneesk., 84 (1940) 1494 146

Halbertsma, Tj., *Een onder sulfanilamide-therapie genezen geval van influenzabacillen-meningitis.* Maandschr. v. Kindergeneesk. VIII (1939) 25 147

Haex, A. J. Ch., and Korthof, G., *Die Wirkung von Dagénan bei meningitis cerebrospinalis epidemica.* Acta brev. neerl. X (1940) 179 146

Hulst, L. A., and Cornelis, J., *Anurie na sulfapyridinebehandeling.* Ned. Tijdschr. v. Geneesk., 84 (1940) 4055 148

Hulst, D. L., *Twee patiënten met endocarditis lenta, genezen respectievelijk door prontosil en cibazol.* Ned. Tijdschr. v. Geneesk. Noodnummer III (1944) 27 145

Jong, J. J. de, *Irgamide en irgafeen. Aanwinsten op het gebied der chemotherapie.* Ned. Tijdschr. v. Geneesk. Noodnummer VI (1944) 60

Jonxis, J. H. P., *De bepaling van de hoeveelheid carbo-, met- en sulfhaemoglobine in kleine hoeveelheden bloed.* Maandschr. v. Kindergeneesk. XI (1942) 204 144

Jonxis, J. H. P., *Over het voorkomen van carbo-, met- en sulfhaemoglobine bij kinderen.* Maandschr. v. Kindergeneesk. XII (1943) 143 144

Jonxis, J. H. P., *De vergiftiging der koolzuuranhydrase door sulfanilamide-verbindingen.* Ned. Tijdschr. v. Geneesk., 87 (1943) 1669; 88 (1944) 227 144

Josso, J., *Klinische ervaringen met sulfamethylpyrimidine bij kinderen.* Ned. Tijdschr. v. Geneesk. Noodnummer XVIII (1945) 204 149

Keiner, G. B. J., *De locale behandeling van het ulcus serpens met dagénan*

Cited on page

(sulphapyridine). Ned. Tijdschr. v. Geneesk., 87 (1942) 3187 . . 146

Keiner, G. B. J., *De plaatselijke behandeling van het ulcus serpens met dagénanpoeder.* Ned. Tijdschr. v. Geneesk., 87 (1943) 252 146

Klessens, J. J. H. M., *Verlammingen in het gebied van den plexis lumbosacralis na intraglutaeale solvochine- en ultraseptylinspuitingen.* Ned. Tijdsch. v. Geneesk., 85 (1941) 2174 148

Knoppers, A. Th., *De antipyretische werking van eenige sulfonamiden bij de rat.* Ned. Tijdschr. v. Geneesk., 86 (1942) 1187 144

Knoppers, A. Th., *The antipyretic action of a few sulphonamides in rats.* Acta brev. neerl. XII (1942) 99 144

Knoppers, A. Th., *L'acide para-amino-benzoïque et l'action antipyrétique de sulfathiazol.* Arch. int. Pharm. Ther., 68 (1942) 470 144

Korthof, G., *Wijziging in de bacteriologische techniek bij onmiddellijke toediening van dagénan.* Ned. Tijdschr. v. Geneesk., 84 (1940) 2793

Kraan, J. K., and Veen, L. van, *Onderzoek over de oorzaak der cyanose na het gebruik van sulfanilamide, sulfapyridine en sulfa-(methyl-) thiazolpraeparaten.* Ned. Tijdschr. v. Geneesk., 86 (1942) 825 . . 144

Kramer, P. H., *Over "spotted fever" (meningococcussepsis) en de behandeling van deze ziekte met sulfapyridine-praeparaten.* Ned. Tijdschr. v. Geneesk., 85 (1941) 2687 146

Kröger-Wibaut, C. M. and Herderschée, D., *Sulfanilamide bij roodvonk.* Ned. Tijdschr. v. Geneesk., 84 (1940) 2341 145

Kuenen, W. A., and Haex, A. J. Ch., *De therapie der meningitis cerebro-spinalis epidemica met dagénan.* Ned. Tijdschr. v. Geneesk., 84 1940 1602 146

Lindeboom, G. A., *De nieuwe chemotherapie in de praktijk.* Amsterdam 1940; sec. ed. 1944 152

Lindeboom, G. A., *Agranulocytose na gebruik van sulfapyridine.* Ned. Tijdschr. v. Geneesk., 85 (1941) 4716 149

Lookeren Campagne, J. van and Westenberg, F. J., *Chemotherapie bij diphtherie.* Ned. Tijdschr. v. Geneesk., 88 (1944) 14 147

Loopuyt, L. and Lubsen, N., *Twee gevallen van chronische longontsteking, veroorzaakt door kapsel-bacteriën en genezen met sulfathiazol. Gewrichtszwellingen als verwikkeling na gebruik van sulfathiazol.* Ned. Tijdschr. v. Geneesk., 87 (1943) 886 147

Lopes Cardozo, P., *Chemotherapie bij bacillaire dysenterie.* Thesis Groningen 1940. Compare Ned. Tijdschr. v. Geneesk., Noodnummer XVI (1945) 183 147

Lopes Cardozo, P., *De behandeling van bacillaire dysenterie met sulfonamides.* Ned. Tijdschr. v. Geneesk. Noodnummer III (1944) 21 148

Lopes Cardozo, P., *Over de thans bestaande specieele darmsulfonamides.* Ned. Tijdschr. v. Geneesk. Noodnummer XIII (1945) 140 . 148, 150

Lopes Cardozo, P., *Euvernil.* Ned. Tijdschr. v. Geneesk., Noodnummer XXVI (1945, a) 309 150

Meer, P. van der, *Clinische ervaringen met sulfa-methylpyrimidine*

(*Percoccide*). Ned. Tijdschr. v. Geneesk. Noodnummer II (1944) 9 149

Meer, P. van der, *De concentratie van sulphanilamideverbindingen, in het bijzonder van sulfamethylpyrimidine (Percoccide), in bloed, liquor cerebrospinalis, urine en pleura-exsudaat.* Ned. Tijdschr. v. Geneesk. Noodnummer XXXII (1945) 407 149

Mulder, J., *Het ontstaan van sulfanilamidopyridine-vaste pneumococcus-stammen in vivo.* Ned. Tijdschr. v. Geneesk., 84 (1940) 923 . . . 146

Mulder, J., *De specifieke behandeling van de door pneumococci veroorzaakte aandoeningen.* Ned. Tijdschr. v. Geneesk., 84 (1940) 1232, comp. 2200 146

Mulder, J., *De prophylaxis en de behandeling van de respiratoire influenza-verwikkelingen met sulfanilamide en derivaten.* Ned. Tijdschr. v. Geneesk., 85 (1941) 482 147

Mulder, J., *Enkele vraagstukken bij de chemotherapie met sulfanilamidederivaten.* Ned. Tijdschr. v. Geneesk., 85 (1941) 4446 . . . 146

Mulder, J., Zoo de Jong, H. H. van der, and Snyman, H. W., *Resorptie en concentratie in bloedplasma en weefselvocht, en uitscheiding van het sulfanilamidopyridine.* Ned. Tijdschr. v. Geneesk., 84 (1940) 2228 143

Munk, J., *Ervaringen met sulfanilamide.* Ned. Tijdschr. v. Geneesk., 84 (1940) 4086 146

Munk, J., *Ervaringen met sulfanilamide.* Maandschr. v. Kindergeneesk. X (1941) 26 146

Nieuwenhuizen, C. L. C. van and Loon, J. A. van, *Enkele kantteekeningen bij het gebruik van sulfapyridine.* Ned. Tijdschr. v. Geneesk., 86 (1942) 890 . . . .

Ormondt, A. van, *Dagénanbehandeling van pneumonieën bij kinderen.* Ned. Tijdschr. v. Geneesk., 84 (1940) 3298 146

Pinkhof, J., *De concentratie van sulfanilamido-pyridine in de vloeistoffen van het oog toegediend bij het konijn.* Ned. Tijdschr. v. Geneesk., 83 (1939) 3711 143

Polano, M. K. and Neering, K., *De chemotherapie van gonorrhoe bij den man.* Ned. Tijdschr. v. Geneesk., 86 (1942) 548 146

Polano, M. K. and Neering, K., *De behandeling met sulfathiazolen van de gonorrhoe van den man.* Ned. Tijdschr. v. Geneesk., 86 (1942) 1312 146

Polano, M. K., *De chemotherapie van gonorrhoe bij den man (III).* Ned. Tijdschr. v. Geneesk. Noodnummer VII (1944) 71 147

Polano, M. K., *De chemotherapie van gonorrhoe bij den man.* Ned. Tijdschr. v. Geneesk. Noodnummer X (1945) 110 147

Prakken, J. R. and Stigter, W., *Koortsbehandeling bij tegen chemotherapie resistente gonorrhoe.* Ned. Tijdschr. v. Geneesk., 86 (1942) 1131 147

Prakken, J. R., Verwoerd, P. J. and Carol, G. A., *Gecombineerde koorts- en chemotherapie bij gonorrhoe.* Ned. Tijdschr. v. Geneesk.,

Cited on page

88 (1944) 64 147

Punt, A., *De invloed van sulfanilamide op de weefseloxydatie in vitro.* Ned. Tijdschr. v. Geneesk., 87 (1943) 1116 145

Quarles van Ufford, W. J., *Over de wondgenezing en hare beinvloeding, in het bijzonder door chemotherapeutica.* Thesis Utrecht, 1945 150

Royer, G., *Prontosil als therapie bij agranulocytose.* Ned. Tijdschr. v. Geneesk., 85 (1941) 1587 147

Ruitinga, P., *Behandeling van infecties der urinewegen.* Ned. Tijdschr. v. Geneesk., 85 (1941) 2153 . . . .

Rijnders, H., *Over de intra-peritoneale toepassing van enkele chemotherapeutica bij de behandeling van diffuse peritonitis.* Ned. Tijdschr. v. Geneesk. Noodnummer XI (1945) 114 151

Rijnders, H., *Over het intraperitoneale gebruik van enkele chemotherapeutica ter vermijding van peritonitis en buikwandettering na colonoperatie.* Ned. Tijdschr. v. Geneesk. Noodnummer XIII (1945) 142 151

Samson, N., *Agranulocytose bij twee zusters.* Ned. Tijdschr. v. Geneesk. 84 (1940) 1623 149

Schoonhoven van Beurden, A. J. R. E. and Loonstra, Tj., *Over de sulfanilamidebehandeling van 22 lijders aan meningococcusmeningitis en de sulfanilamido-pyridinetherapie van 4 lijders aan pneumococcusmeningitis.* Ned. Tijdschr. v. Geneesk., 84 (1940) 3673 146

Snapper, I., Liu, S. H. and Chung, H. L., *Haematurie, niercoliek en acetyl-sulfapyridine-steenvorming na sulfapyridinebehandeling.* Ned. Tijdschr. v. Geneesk., 83 (1939) 4501 148

Stigter, W., *De behandeling van gonorrhoe met dagénan.* Ned. Tijdschr. v. Geneesk., 85 (1941) 2490 146

Sutorius, J. E. M., *Een geval van anurie tengevolge van dagénan.* Ned. Tijdschr. v. Geneesk., 84 (1940) 4948 148

Sutorius, J. E. M., *Een geval van anurie tengevolge van dagénan.* Ned. Tijdschr. v. Geneesk., 85 (1941) 1893 148

Sutorius, J. E. M., *Chemotherapie bij gonorrhoe.* Thesis, Groningen (1941) 148

Tilanus, A. D. W., *Rectale sulfapyridinetoediening.* Ned. Tijdschr. v. Geneesk., 86 (1942) 1953 144

Trap, Y. M., *Pneumonie van drie longkwabben, onder invloed van Dagénan abortief verloopen.* Maandschr. v. Kindergeneesk. IX (1940) 31 . . . .

Veen, G. van, *Behandeling van etterige huidinfecties met sulfanilamidothiazol.* Nederl. Tijdschr. v. Geneesk. Noodnummer XXXI (1945) 366 145

Velde, L. H. van der, *Een geval van erythema exsudativum multiforme, genezen met sulfanilamide (prontosil album).* Ned. Tijdschr. v. Geneesk., 84 (1940) 3085 147

Cited on page

VERHAGEN, H., *Het sulphapyridine. Klinische en biochemische onderzoekingen.* Thesis, Amsterdam (1941) 143

VERHAGEN, H., *Onderzoekingen over sulfamethylthiazol en sulfamethylpyrimidine.* Ned. Tijdschr. v. Geneesk., 86 (1942) 1171 143

VERHAGEN, H., *Pharmacologische onderzoekingen over het sulfamethylthiazol (ultraseptyl) en sulfa-methylpyrimidine (percoccide) vergeleken met het sulfapyridine (dagénan), benevens enkele clinische ervaringen.* Ned. Tijdschr. v. Geneesk., 86 (1942) 3048 143

VERHAGEN, H., *Over het verband tusschen klinisch effect en de pharmacologie van een aantal sulfanilamide-derivaten.* To be published in Geneesk. Bladen; and more detailed in Acta Med. Scand (A) 151

VISSER, A., *De behandeling van erysipelas met sulfanilamide.* Maandschr. v. Kindergeneesk. VIII (1939) 25 145

VLEUGELS SCHUTTER, G. J. N., *Morbus Duhring behandeld met dagénan.* Ned. Tijdschr. v. Geneesk., 85 (1941) 2618 147

WALDECK, K., *Het gebruik van het chemotherapeuticum euvernil in de urologie en chirurgie.* Ned. Tijdschr. v. Geneesk. Noodnummer XIV (1945) 154 150

WEERSMA, M., *De behandeling van meningococci- en van pneumococcimeningitis met dagénan.* Ned. Tijdschr. v. Geneesk., 84 (1940) 1610 146

WESTERHOF, M. E., *Uitkomsten van de behandeling van gonorrhoe met dagénan.* Ned. Tijdschr. v. Geneesk., 85 (1941) 434 146

WEY, A. P. VAN DER, *De behandeling van bacillaire dysenterie met sulfonamides.* Ned. Tijdschr. v. Geneesk. Noodnummer VII (1944) 74 150

WEYERS, W. A. M., *Influenzabacillen-meningitis en dagénan.* Ned. Tijdschr. v. Geneesk., 83 (1939) 4800 147

WIELENGA, D. K., *Post-traumatische shock en gasgangraen met goed gevolg behandeld met herhaalde groote bloedtransfusies en sulfanilamide.* Ned. Tijdschr. v. Geneesk., 83 (1939) 4740 147

ZELDENRUST, J. AND VERLINDE, J. D., *Over het voorkomen van necroses en de oorzaak van den dood bij pneumonieën, welke met sulfapyridine zijn behandeld.* I and II. Ned. Tijdschr. v. Geneesk., 86 (1942) 2520, 2574 145

ZELDENRUST, J. AND VERLINDE, J. D., *Over doodelijk verloopen pneumonieën, welke met sulfapyridine zijn behandeld.* Ned. Tijdschr. v. Geneesk., 86 (1942) 2880 145

ZOO DE JONG, H. H. VAN DER, *Estimation of sulfapyridine.* Lancet (1939) 833 143

V. MYCOTHERAPY, RESEARCHES ON ANTIBIOTIC SUBSTANCES

(BY J. J. DUYVENÉ DE WIT)

§ I. EXPANSINE

In the Netherlands, too, an extensive research-group[1], during the war, has made investigations on the isolation of antibiotic substances from the media of micro-organisms and on the possibility of their practical application. These investigations, however, did not originate from the well-known discovery by Fleming (1929), which was the starting-point of the English and American investigators, but from the work of van Luijk (1938, 1939).

As early as 1932 van Luijk, in the Phytopathological Laboratory "Willie Commelin Scholten" (Baarn), commenced a comprehensive study of the antagonistic action of micro-organisms against plant-parasites, and in 1939 he pointed out the possible importance of metabolic products of micro-organisms in medical therapeutics.

Van Luijk commenced his work, actuated by the following interesting question: Why is it that, in an infected soil, some of the plants perish as the result of infection, whilst others, equally exposed to the same infection, remain healthy? He suggested that probably the presence of some antagonistic micro-organism inhibited, completely or in part, the activity of the pathogenic micro-organism.

---

[1] *Microbiology:* A. van Luijk, Botanisch Laboratorium Rijksuniversiteit Utrecht, Director: Prof. Dr. V. J. Koningsberger.
Miss A. Jaarsveld, Bacteriologisch Laboratorium Gemeentelijke Geneeskundige en Gezondheidsdienst, Amsterdam, Head: Prof. Dr. A. Ch. Ruys.
Chemistry: H. K. Oosterhuis and R. Luyken, Laboratorium voor physiologische chemie, Gemeentelijke Universiteit and Nederlandsch Instituut voor Volksvoeding, Amsterdam Director: Prof. Dr B. C. P. Jansen.
W. Th. Nauta, J. W. Dienske, A. van der Linden, P. van Duyn, J. Engelsman and Miss A. C. Faber, Scheikundig Laboratorium Vrije Universiteit, Amsterdam, Director: Prof. Dr Ir J. Coops.
Pharmacology: A. M. Ernst, Pharmacologische Afdeeling Kliniek voor kleine huisdieren Rijksuniversiteit Utrecht, Director: Prof. Dr A. Klarenbeek.
Medical applications: J. D. Verlinde, Instituut voor praeventieve geneeskunde, Leiden Director: J. P. Bijl.
A. J. J. Bosma, Dermatologische Afdeeling Academisch Ziekenhuis, Leiden.

In order to test the truth of this hypothesis, VAN LUIJK isolated the micro-organisms in the soil around the healthy plants. From these, Penicillium expansum proved to be the most active. Making use of the extensive collection of the "Centraal Bureau voor Schimmelcultures"[1] he investigated quite a series of fungi to determine their antagonistic action towards those fungi which were pathogenic to plants. As a result of these investigations, certain strains of Penicillium expansum (Link Thom.) seemed especially capable of producing antagonistic substances. The sensitivity of the various pathogenic fungi towards the active agent, however, proved to be very variable. From those varieties which cause the rot of roots and stems, the growth of various strains of Rhizoctonia and of Thielavia Basicola was completely inhibited by strong dilutions of culture liquid taken from a growth of P.expansum. Many species and varieties of Fusarium, however, proved to be very resistant in this respect.

In 1938 VAN LUIJK was able to demonstrate that a strongly antagonistic action of P.expansum was especially noticeable towards various species of Pythium, which caused black root rot in some graminaceae and in lucerne. One case was observed in which the growth of Pythium de Baryanum Hesse in a culture liquid of P.expansum, diluted 1280 times, was completely prevented even after 5 days. In the most marked of these cases, the activity of the culture liquid was equal to that of 0,02 per cent mercuric chloride solution. Also in the case of Trichophyton Rosaceum Sab., a fungus pathogenic to man, a similar result proved to be obtainable; indeed, it was shown that the addition of minute quantities of the culture liquid of P.expansum was sufficient to prevent growth completely.

The anti-biotic factor proved to be thermo-stable and capable of extraction from an aqueous solution with chloroform. After adsorption to norit and elution with ether, 70–80 % of the active agent in a considerably purified state could be recovered. To this agent VAN LUIJK gave the name "Expansine".

---

[1] Director: Prof. Dr JOHA. WESTERDIJK, to whom we are much indebted for her valuable aid and the interest received for our work.

VAN LUIJK also isolated other micro-organisms of strongly antibiotic action.

In agriculture and horticulture, disinfection of sowing seed, which is always infected with bacteria, is carried out more and more. The dressings employed to do this are not entirely harmless, however, since they have to be employed in rather high concentrations to be effective, and VAN LUIJK, therefore, considered it important to try treating the seeds with the culture liquid of P. expansum. This proved to be very effective.

WYBRANS is now studying the effectiveness of antibiotic substances in fighting mould diseases affecting plants of economic importance.

In view of these facts, the question arose of whether it would be possible to isolate from a culture of P.expansum one or more substances pre-eminently fit for killing fungi and bacteria pathogenic to man and animal. In order to answer this question, VAN LUIJK (1939) proceeded to isolate a number of strains of P. expansum from garden mould and to test their antibiotic action on Pythium de Baryanum, the fungus which is still used as a testing agent. Antibiotic activity is based on the inhibitory action towards Pythium, and the Antibiotic Index (A.I.) denotes the dilution required just to bring about inhibition of growth. Difficulties, arising in the estimation because of the varying sensitivity of the test agent, may be overcome for the greater part by making use of a standard fungicide, e.g., phenol.

In the most successful isolation experiments of P.expansum, an A.I. of 1 : 2400 proved to be attainable after 10 days' growth in a solution according to KNOP. The use of calcium and iron in the culture medium is essential. In using the media according to RAULIN, KAUFMANN and FLEMING, formation of antibiotics scarcely occurs, neither does it in a medium according to KNOP, prepared with one tenth of the indicated amount of calcium nitrate. As the yield of expansine decreases in pure cultures in the course of a few months, it has proved to be necessary continually to isolate new strains of high productive capacity (SAMUELS BRUSSE). However, Penicillium claviforme has proved to be much more constant in the production of antibiotics and for this fungus too; Knop's culture liquid is the most favourable one.

In addition to the test method mentioned, VAN LUIJK has elaborated another one which enables a quick estimation of antibiotic action to be made (see Fig. 31).

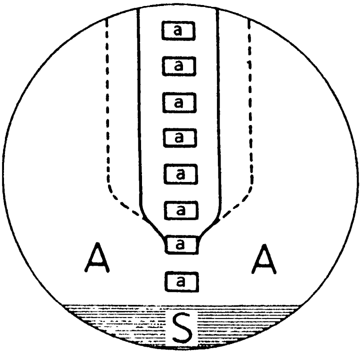


Fig. 31.

AA, Agar-plate (1½ proc.)
S, Segment with nutritious substances ("Knop" - 2½ % saccharose), inoculated with *P. claviforme.*
aa, Cubes, punched from an agar-plate ("Knop" - 2½ % saccharose), inoculated with *Pythium mamillatum.*
Full line, growth of *Pythium* after 24 hours.
Dotted line, growth of *Pythium* after 48 hours.

A Petri dish contains pure agar as a medium; a small segment S, replaced by agar containing nutritious substances, is inoculated with the micro-organism to be tested. Perpendicular to this inoculation-zone and along the central line of the dish, small cubes of agar, inoculated with *Pythium*, are placed at a short distance from one another. This fungus will show a tendency to spread over the agar. Antibiotics, however, if produced by the organisms being tested, will diffuse into the agar and reach the *Pythium mycelia*, preventing or inhibiting their further growth depending upon the degree of their activity. In the case of a positive result, a beautifully clear zone is observed in the course of a few days, serving as an indication of the antibiotic effect. This method allows of numerous variations. For instance, parallel to the series of *Pythium* agar cubes, another series may be placed, provided with an indicator, the colour of which tells something of the reaction of the diffusing substances.

The crude culture medium, containing the metabolic products of P.expansum, is not in itself suitable for therapeutic application. For this purpose it is necessary to concentrate and to purify the agent and therefore its isolation had to be attempted. After van Luijk had shown that this anti-Pythium substance was but little influenced by boiling, could be transferred into lipid-solving media and adsorbed to norit, Oosterhuis and Luijken succeeded in isolating a crystalline substance by chromatographic purification, adsorption to pre-treated norit, elution and crystallisation from a concentrated ethereal solution. In the Pythium test this substance proved to possess an A.I. of 1 : 6.000.000; towards Staphylococcus aureus it still acts in a dilution of 1 : 100.000. This substance was called expansine (Duijvené de Wit and coll., 1944).

In the same way, claviformine was shown to be obtainable from a culture medium of P.claviforme and the two substances proved to be identical. They have the same melting point, the same mixed melting point and an identical mixed chromatogram of the phenyl-hydrazones.

Chemical data.

The molecular formula of expansine, after being thought to be $C_9H_8O_5$, proved to be $C_7H_6O_4$ (Nauta, Oosterhuis, van der Linden and van Duijn).

The melting point is 110–111° C, at which temperature it becomes yellow in colour.

It is optically inactive and forms stable solutions in most solvents, e.g., in acetone, benzene and pea-nut oil.

The aqueous solution is neutral when freshly made, but after some days when tested with Congo Red shows the presence of strong acid.

A freshly prepared aqueous solution gives no coloration with ferric chloride, colours a fuchsin-sulphur dioxide solution after some hours, reduces an ammoniacal silver solution in the cold and Fehling's solution on heating.

Legal's reaction with nitroprusside and a drop of piperidin is strongly positive.

The solution, after standing for some time, shows the coloration characteristic of enols on addition of ferric chloride.

An aqueous solution of expansine, on adding 2n NaOH, develops a bright yellow colour which, on acidification, disappears shortly afterwards, the solution then giving a positive reaction with ferric chloride.

On standing for a prolonged time, an alcaline solutions turns brown and this coloration no longer disappears on acidification. Heating with NaOH solution produces a volatile substance with a faint smell of acrolein; in this reaction, a molecule of expansine takes up exactly two molecules of NaOH. However, titration in the cold is impossible.

Expansine in solution readily absorbs bromine, varying from one to three molecules depending on experimental conditions.

On catalytic reduction, one to two molecules of hydrogen are absorbed quickly without marked difference in speed, a third molecule is absorbed more slowly. The resulting product consists mainly of an acid.

Titration with hydroxylamine reveals the presence of a carbonyl group; another molecule of hydroxylamine is taken up very slowly.

Expansine forms a monophenylhydrazone, p-nitrophenylhydrazone, 2,4-dinitrophenylhydrazone and a semicarbazone. Compounds can also be formed with dimethone and benzaldehyde. All these substances are inactive in the Pythium test.

By means of Zerewitinoff's method, one interchangeable hydrogen atom is found.

A primary or secondary alcoholic function seems to be present, as is shown by a strongly positive reaction with diazotized sulphanilic acid (alcaline medium) and a slow reaction with nitrochromic acid (strongly acid medium). With Stoecklin's reagent in a weak acid medium the reaction on a primary alcoholic group is negative, however. One should take account of the possibility that, under the conditions of the reactions, expansine is not stable.

With acid chlorides, the formation of esters is possible. The following were prepared (DIENSKE) and partly tested as to their activity:

acetate:	m.p. 115—117°
benzoate:	,, 141,5°
p-nitrobenzoate:	,, ca. 230° (decomp.)
p-acetylaminobenzenesulphonate:	,, 117—118°
cinnamate:	,, 122—123°

Ozonization yields CO_2, formic acid and a complicated mixture of ketones, partly with acidic character. Glycol aldehyde was isolated as its dimethone derivative. Separation of the ketones by chromatographic absorption of the p-nitrophenylhydrazones seems possible.

By decomposition with acids CO_2, formic acid and methylethylglyoxal: $CH_3.CO.CO.CH_2.CH_3$ are formed.

The determinations of methoxyl and carbonmethyl groups in expansine gave negative results.

By heating expansine in hydrochloric acid with phloroglucinol an additive compound of the molecular ratio 1 : 1 is quantitatively formed.

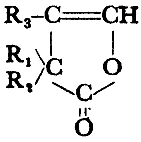
From these data the following deductions can be made about the structure of expansine:

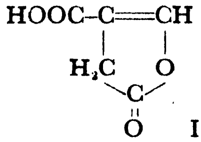
1. Expansine contains a lactone ring; as this ring is only slowly opened to an acid, expansine is probably a γ-lactone.

2. The coloration with NaOH, and the reduction of ammoniacal silver solution indicates an unsaturated γ-lactone, which contains its double bond inside the ring.

3. The catalytic reduction to an acid, under absorption of two moles of hydrogen without marked difference in speed, is possible only in the case of a γ-lactone derived from an enolized oxo-acid, which, in this case, means: an unsaturated γ-lactone with the double bond in β, γ-position.

4. The acid obtained by opening of the ring gives colorations with ferric chloride and fuchsine-sulphur dioxyde solution. This is possible if the γ-carbon atom of the lactone ring is unsubstituted; that means that expansine is a lactone of a β-aldehydo acid and thus contains the structure I:

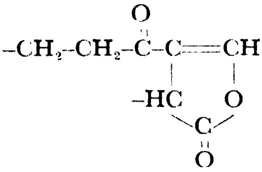

I

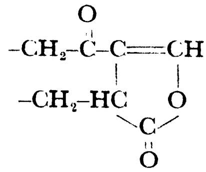

II

The formation of a dimethone-derivative and of formic acid by the ozonization finds here also an explanation. Aconic acid (II) in general shows the same reactions.

5. The formation of methyl-ethyl-glyoxal reveals the presence of a normal chain of five carbon atoms in expansine.

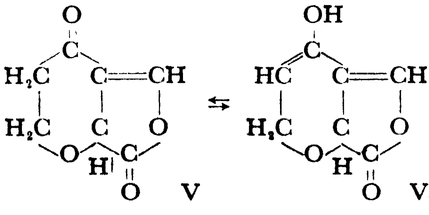
6. As expansine contains one free carbonyl group, the second carbonyl group of the methyl-ethyl-glyoxal must be formed by hydrolysis of a double bond. One of the two CO groups is furnished by the β-carbon atom of the lactone ring. From this it follows that expansine contains the structure-element III or IV:

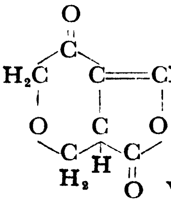

III


IV

These structures also explain the formation of formic acid and CO_2 as products of the hydrolysis by acids (the latter originating from the primarily formed β-keto-acid).

7. It follows as a matter of course from the amount of hydrogen available in the molecular formula that the fourth oxygen atom should be present as the closing element of the structure III or IV to a cyclic ether (tetrahydropyrone structure). Therefore we find for expansine the formulas V or VI:


V Va


VI

The formation of a benzal compound suggests that V is the most probable structure, it is possible that Va also plays a part.

Raistrick and coll. (1943) isolated a substance $C_7H_6O_4$ as a metabolic product of P.patulum Bainier, which they called patuline. According to these authors patuline has probably also the structure V (anhydro-3-hydroxymethylene-tetrahydro-4-pyrone-2-carboxylic acid). As the original literature was not available, only a few properties of the substances could be compared. We believe expansine, patuline, clavacine and claviformine to be identical (compare CHAIN and coll., 1944; HOOPER and coll., 1944). In order to prove the correctness of the structure assigned to expansine, a synthesis is in progress. Part of the investigations mentioned above were hampered by the impossibility of carrying out analyses, caused by the lack of sufficient supplies of gas and electricity.

Pharmacological data.

An orienting study of the pharmacology of expansine was made by ERNST. Remarkable is its relatively high toxicity. The lethal dose (intravenous injection) in mice, cats and rabbits varies from 10–15 mg per kg of body weight. In dogs 2,5 mg of expansine per kg of body weight, administered intravenously, are tolerated without clinical symptoms, while 5 mg per kg exerts a distinct, temporary effect, in the form of tachypnoea and a negative T-wave in the electro-cardiogram.

In mice the subcutaneous or intramuscular injection of 5 mg of expansine in one ml of 0,9 % NaCl solution produces a local inflammatory reaction.

In cats, the isolated heart, isolated intestine, the respiration, reflex function and blood pressure are still fatally influenced in concentrations of 1 : 5000; 1 : 15000; 1 : 9000; 1 : 9000 respectively.

Activity in vitro and in vivo.

The investigation on the antibiotic action of the culture medium of P.expansum by Miss JAARSVELD (1940, 1942), showed that the crude concentrate as well as the purified product, is indeed, active against various micro-organisms pathogenic to man.

This activity can be read from Table VIII. Several strains from each micro-organism were tested.

TABLE VIII

Micro-organism	Minimal lethal dose of culture medium of P.expansum		Minimal lethal concentration of pure expansine, in mg/l
	expressed as A.I.	activity converted in expansine, expressed in mg/l	
Corynebacterium diphtheriae			
typus mitis	340–1215	60–200	
typus intermedius	1215–3400	200–600	
typus gravis	160–3400	25–600	100–500
Bacillus anthracis	60	10	
Staphylococcus aureus	300	50	500
Hemolytic streptococci	60	10	
Brucella abortus	300	50	
Brucella melitensis	60	10	
Eberthella typhosus	60–300	10–50	100–500
Escherichia coli	300	50	100–500
Proteus vulgaris	300	50	
Pseudomonas aeruginosa	300	50	
Salmonella Schöttmuelleri	300	50	100–500
Shigella dysenteriae	300	50	
Shigella paradysenteriae	60–300	10–50	500
Shigella Sonnei	60	10	100–500
Vibrio comma	6	1	50
Mycobacterium tuberculosis			
typus bovinus	30–300	5–50	
typus humanus	300	50	
Trychophyton interdigitale	30–300	5–50	

A solution of pure expansine proved to be less active towards most of the micro-organisms tested than a culture medium containing the same amount of antibiotic substance (expressed as A.I.). This can be read from Table VIII by comparing the figures in columns 2 and 3.

The same becomes evident if, on account of the known minimal lethal concentrations of pure expansine for Pythium (0,2 mg/l) and for Staphylococcus aureus (10 mg/l), the anti-

Staphylococcus activity is calculated from the measured anti-Pythium activity. The activity against Staphylococci then often proves to be distinctly lower than the value determined experimentally. This may mean that besides expansine, one or more other antibiotics are present in the culture medium. Indeed, after the removal of expansine, the mother liquid still proved to possess distinct antibiotic properties. Attempts were made to identify the antibiotic factors involved. By means of chromatographic adsorption OOSTERHUIS succeeded in isolating three more substances from the culture liquid of P.expansum, two of which possess strongly reducing properties. These substances have not yet been identified, but are devoid of any antibiotic activity.

The activity of expansine in vivo was investigated by VERLINDE. Orienting tests on experimental infections with Staphylococci, Leptospira icterohaemorrhagiae, Clost. Welchii, Streptococcus haemolyticus, Pneumococci, Salm. typhi murium, Corynebacterium diphtheriae, Mycobacterium tuberculosis and the viruses of vaccinia, influenza and herpes produced negative results.

Nothing can be said with any certainty about the way in which expansine exerts its antibiotic effect. This action is inhibited by serum. On being introduced into the rat's stomach it is inactivated. Expansine causes no hemolysis. Investigations by WILLEBRANS show that the substance is able to influence various enzymic processes. Expansine possesses an anti-urease activity, but the activity remaining in the mother liquid is so strong, that probably another factor, of a much stronger anti-urease activity, is present.

Therapeutic applications.

Not only chemically, but also therapeutically expansine is considerably different from penicilline. Owing to its toxicity it can be applied only locally, thus reducing consideration of its therapeutic application to the treatment of bacterial skin affections, and principally of skin mycoses, as the substance possesses a distinct activity towards pathogenic fungi of the species Trichophyton, Achorion and Epidermophyton.

In the beginning these affections were treated by pencilling with an ethereal solution. After that a vaseline ointment, containing expansine, was applied and, finally, the best results were found to be obtained if a mixture of adeps lanae and adeps suillus (1 : 1) was used as a basis. Some dermatologists have successfully applied this ointment in various diseases of the skin.

Prakken applied expansine to twelve patients suffering from skin mycosis. At first an ethereal solution was used. Though the treatment with this solution has the distinct advantage, especially in wartime, of being applicable without the use of bandages and not soiling the clothing, strong irritations were observed. For that reason it was afterwards replaced by the expansine ointment mentioned above.

The reports concerning a number of patients are as follows:

1. Woman aet. 45. Had several years' history of typical Athletes Foot. There were extensive lesions on the sole of one foot and between the toes. Some infection was also observed on the finger nails, and in some areas this infection was observed to be of an exsudative nature. Tests for fungi were positive for Epidermophyton. Treatment was by expansine in ethereal solution of A.I. 1 : 4000. After three days the treatment had to be interrupted on account of the very marked irritant reaction produced.

2. Woman aet. 19. This woman had an extensive Epidermophytotic infection which had been regularly treated with the recognized antimycotic preparations without even preventing spread of the infection. Material from the nails was positive for the fungus test: typical Epidermophyton. After five weeks treatment with expansine in ethereal solution (A.I. 1 : 6000), there was a very considerable improvement which was maintained with a less concentrated solution, viz. A.I. 1 : 2000. On inspection three months after the start of treatment with expansine, apart from desquamation and erosion between the toes, the lesions were completely healed. The treatment of this interdigital condition was continued with Ung. expansine (A.I. 1 : 10.000). At this juncture the war interrupted the supervision and treatment but, from a letter received 5 months later, it would appear that the condition has relapsed, no doubt because treatment was not maintained for a sufficiently long period.

3. Boy aet. 16. This boy, handling infected cattle in his farmwork, was suffering from lesions on the arms, shoulders and legs suggestive of Trichophyton superficialis. The fungus test was positive. Treatment with ethereal expansine (A.I. 1 : 25.000) produced, at the end of seven days, a distinct improvement but there was evidence of too much

irritation. The treatment was continued with ethereal expansine (A.I. 1 : 20.000). The redness and desquamation continued, so Ung. expansine (A.I. 1 : 10.000) was substituted. This was tolerated very well and, at the end of seven weeks, healing appeared to be complete. As a precaution however, a few red looking spots were treated with Ung. expansine (A.I. 1 : 8000) and a month later these too had healed.

4. Girl aet. 16. Clinically this girl had a typical Epidermophyton infection around the toes of both feet. Tests for fungus were positive for typical Epidermophyton. There was a clinical cure in 2 weeks after using Ung. expansine (A.I. 1 : 12.000).

From the above-mentioned patients, the fungi were cultured and classified at the Centraal Bureau voor Schimmelcultures at Baarn.

Prakken arrived at the conclusion that expansine is a valuable "anti-mycotic", but that the necessary data concerning the optimum strength and method of application and the more precise indications are still lacking.

Bosma applied expansine to a series of cases:

Epidermophyta		8
Trichophyta	superficiales	6
,,	profunda	14
,,	of the nails	5

Unfortunately, owing to the war, treatment and observation were not as thorough or as intense as he would have wished. When the affection did not heal, it was the irritant properties of the preparation which were to blame. Below some of these cases are detailed:

1. Woman aet. 25. Typical Epidermophyta of the palmar and dorsal surfaces of both hands. Fungus test was positive. Treated with Ung. expansine (A.I. 1 : 6000), for three days with improvement, but papules and vesicles were still present and the fungus test still positive at the end of this time. Four days later, Ung. expansine (A.I. 1 : 40.000) was applied but the patient went on holiday for two weeks and did not continue treatment. The fungus test was again positive. However, another seven days' intensive therapy produced a marked improvement, the red rims of the lesions had disappeared and the fungus test was negative. At the end of another fourteen days, the condition was healed and the skin smooth. No further fungus could be found.

2. Man aet. 27. Epidermophyta of the fingers and palm of the right hand. The affected areas were scaly, vesicular and dry; in some places there were brownish spots and rhagades. The fungus test was positive.

The patient gave a history of frequenting indoor swimming pools, and the affection had been present for four months. The treatment was with ethereal expansine (A.I. 1 : 6000) four times a day. There was improvement in 48 hours, but the fungus test was still positive. Three days later, the activity was greatly reduced but the periphery of the lesions were still vesicular. These vesicles were less prominent in another 48 hours, and the fungus test negative. The strength of the solution was reduced to A.I. 1:2000 but, after a week, this had to be stopped and Ung. Zinc. applied to reduce the irritation; only a few scaly spots remained after four days, when the fungus test was again negative. Ten days later, however, the lesions had reappeared and the fungus test had become positive. Ethereal expansine (A.I. 1 : 40.000) was resumed but, in another six days, vesicles were found and an anidrotic eczema had appeared so that it seemed advisable to continue the treatment with a 20 proc. zinc-vaseline ointment.

3. Woman aet. 67. Large area of Epidermophytosis of the sole and the interdigital spaces of the right foot. The fungus test was positive. The treatment was ethereal expansine (A.I. 1 : 6000). Three days afterwards the squamous scales disappeared and new skin appeared in its place. The fungus test was still positive, however. Treatment was continued with expansine solution (A.I. 1 : 20.000) and after five days, the condition was greatly improved. The fungus test was now negative and after a further three days' treatment with expansine (A.I. 1 : 40.000) the condition was very markedly improved. Two days later, new vesicles appeared on the foot but the fungus test was negative. The lesion was completely cured after one month and no trace of fungus could be found.

4. Girl aet. 15. A case of typical Epidermophytosis of the right foot and interdigital spaces with a positive fungus test. Treatment was with ethereal expansine (A.I. 1 : 40.000). After ten days, there was a marked improvement in the condition of the skin, and after a further four days there was complete healing with a negative fungus test.

Of the six patients suffering from Trichophyton superficialis, five were cured; the sixth was definitely improved but treatment with expansine caused irritation, and had to be interrupted:

1. Boy aet. 7. On the left thigh there was a lesion of the erythemato-squamous type. Diagnosis: Trichophyton superficialis. The fungus test was positive. The condition had been treated by the family doctor for one week with Iodine without success. Treatment with ethereal expansine (A.I. 1 : 6000) was begun and, after one day, the lesion was less scaly with the skin at the periphery beginning to peel off. The fungus test was positive. The following day, healthy new skin appeared and the fungus test was negative. After a further six days' treatment, the periphery of the lesion was quite smooth and only a few crusty

scales remained. The fungus test was negative. Three weeks after this, the patient was completely cured and no trace of fungi could be found.

2. Man aet. 32. On the face and neck there were the typical erythematosquamous lesions of Trichophyton superficialis. These were treated with ethereal expansine (A.I. 1 : 40.000). The fungus test was positive. After ten days' treatment on these lines, the skin was almost entirely smooth, there were no fungi present and, after a further seven days, the patient had completely recovered.

3. Man aet. 62. The lesion was a typical Trichophyton superficialis Barbae of the left lower jaw region. The fungus test was positive. Ung. expansine (A.I. 1 : 12.000) was applied and four days later there was only the slightest trace of a ring remaining. After seven days, this was found to be spreading slightly towards the lower jaw again, and Iodine was therefore applied. After the lapse of a further eight days, the skin looked quite sound. Three days' treatment with a 5 per cent sulphur salicyl. in vaseline ointment completely cured the condition.

None of the fourteen young patients, all inmates of a Childrens' House, who were found to have a Trichophyton profunda infection, was healed. In the course of the treatment with expansine in ethereal solution, which was carried out by the nurse, it was, however, noticed that the children did become alternately fungus-negative and -positive. After a clinical cure of seven weeks' duration, the fungus test was negative in eleven cases. One week later, during which time no treatment was given, all of them were fungus-positive again. Röntgen epilation and Iodine therapy brought about definite healing subsequently.

The treatment of five patients, suffering from Trichophytosis of the nails of the fingers, did not produce healing either. However, owing to the circumstances, the treatment could not be carried out sufficiently long, but even so a slight improvement could be observed in some of these cases.

1. Girl aet. 19. The nail of the right middle finger was brittle, showing in addition pits and white spots. Diagnosis: Trichophytosis. Fungus test positive. Therapy: ethereal expansine solution (A.I. 1 : 6000). The nail was scraped and appeared to be much improved after fourteen days, although the fungus test was still positive. After a further fourteen days, the nail became more glossy but fungi were still present, and after the lapse of an additional seven days the nail looked beautiful and there were no fungi. After the next seven days, however,

the palms of the hand showed erythemato-squamic spots and were treated with Ung. expansine (A.I. 1 : 20.000).

For the purposes of control, one patient suffering from Lupus was treated:

1. Man aet. 54. The forearm showed a red-purple, crusty, pustulous, scaly and papulous area measuring 6 × 2½ cm. Diagnosis: Lupus vulgaris. Therapy: pencilling three times a day with a concentrate from a culture medium of Penicillium expansum (A.I. 1 : 20.000). After seven days' treatment the area was very crusty and brownish-yellow in colour. The crust was removed. Fourteen days after this again, a large crust was removed, leaving a very healthy-looking skin at the periphery. In the centre there was a somewhat wet-looking, ulcerating spot which, after two weeks, looked very healthy, no specific inflammatory reaction being provocable. The patient did not return for further observation.

BOSMA arrived at the conclusion that expansine could definitely be considered as a valuable and important therapeutic agent in skin infections especially in superficial mycoses, which react very favourably to expansine. However, the optimum concentration and the most suitable vehicle are still to be determined. Such a vehicle must be capable of carrying the expansine into the follicles and even deeper.

As the formation of antibiotic factors appears to be a property of a great many micro-organisms, the question arises of whether there may be, amongst these substances, some of even greater therapeutic interest than penicillin, here, for example, we think of compounds which are non-toxic to man but lethal to the etiological agents of tuberculosis, diphtheria and typhoid fever. The investigation of this domain implies the testing of a large number of micro-organisms to determine their specific action towards pathogenic bacteria. These investigations, in view of the general interest in antibiotic substances, will certainly be made everywhere together with attempts to isolate the active substances concerned. VAN LUIJK intends to be guided in his investigations by the supposition, similar to that already discussed in regard to micro-organisms of the soil, namely that the healthy skin owes its condition to an antibiotic micro-flora which occurs on it and which produces substances which prevent the intrusion of pathogenic micro-organisms or keep the pathogenic flora within bounds, i.e., preserve a biological equilibrium. Furthermore, he presupposes the presence of an antibiotic flora in the buccal cavity and in the intestine, this flora secreting substances which act locally as well as more remotely.

LITERATURE

Cited on page

Bosma, A. J. J., Unpublished 170

Chain, E., Florey, H. W. and Jennings, M. A., *Identity of patuline and claviformine*. Lancet, 246 (1944) 112; C. (1944) II, 25 . . . 166

Dienske, J. W., Unpublished 163

Duyvené de Wit, J. J., Jaarsveld, A., Jansen, B. C. P., Luijk, A. van, Luyken, R., Oosterhuis, H. K. and Wybrans, J. R., *De isoleering van een bactericide en fungicide stof uit een penseelschimmel.* Ned. Tijdschr. v. Geneesk., 88 (1944) 718 162

Ernst, A. M., Unpublished 166

Fleming, A., *On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of B. influenzae.* Brit. J. exper. Pathol., 10 (1929) 226 158

Hooper, I. R., Anderson, H. W., Snell, P. and Carter, H. E., *The identity of clavacine with patuline.* Science, 99 (1944) 16; C. (1944) II, 25 166

Jaarsveld, A., *De invloed van verschillende bodemschimmels op de virulentie van Rhizoctoria solani Kuhn.* Thesis Amsterdam (1940), Comp. Phytopathol. Ztschr., 14 (1942) 1 166

Luijk, A. van, *Antagonism between various micro-organisms and different species of the genus Pythium, parasitizing upon grasses and lucerne.* Med. Phytopathol. Lab. W. C. Scholten, 14 (1938) 43 . . 158, 159

Luijk, A. van, *Antagonisme tussen micro-organismen.* Vakblad v. Biologen, 20 (1939) 177 . . . . 158, 160

Luijk, A. van, Unpublished 161

Nauta, W. Th., Oosterhuis, H. K., Linden, A. van der, Duyn, P. van, Unpublished. Compare Preliminary note: Nauta, W. Th., Oosterhuis, H. K., Linden, A. C. van der, Duyn, P. van and Dienske, J. W., *The structure of expansine, a metabolic product of Penicillium expansum Westl., with antibiotic properties.* Rec. trav. chim., 64 (1945) 254 162

Oosterhuis, H. K. and Luyken, R., Unpublished 162

Prakken, J. R., Unpublished 169

Raistrick, H., Birkinshaw, J. H., Michael, S. E. and Bracken, A., Lancet, 245 (1943) 625. Cited from Chemie, 57 (1944) 79

Cited on page

Renseignements Scient. Croix Rouge Nr. 2, 44 (1944); Nachr. f. Aussenhandel Nr. 134 (1944) 165

Samuels Brusse, E., Unpublished 160

Verlinde, J. D., Unpublished 168

Willebrans, Unpublished 168

Wybrans, J. R., Unpublished 160

MONOGRAPHS ON THE PROGRESS OF RESEARCH IN HOLLAND DURING THE WAR

EDITORIAL BOARD

R. Houwink, Delft; J. A. A. Ketelaar, Amsterdam

13. Pharmacological Investigations by A. Th. Knoppers.
14. The Rheology of Suspensions by H. de Bruyn.
15. The Wet Purification of Coal Gas and Similar Gases by the Staatsmijnen-Otto-Process by H. A. J. Pieters and D. W. van Krevelen.
16. Theory and Numerical Calculation of Radiowave Propagation around a Spherical Earth by H. Bremmer.
17. Some Aspects of Luminescence in Solids by F. A. Kröger.
18. Theoretical and Experimental Investigations on Radio-Systems with Frequency-Modulation by B. van der Pol.
19. New Developments in Ferromagnetic Materials by J. L. Snoek.
20. Progresses in the Theory of the Physical Properties of Glasses by J. M. Stevels.
21. Sound-Reproduction by R. Vermeulen.
22. The Influence of the Atmosphere and the Heat Treatment on Electrical Welds by H. G. Geerlings.
23. Sound Absorbing Materials by C. Zwikker.
24. Modern Cancerology by N. Waterman.

Other volumes to follow